

ANALYZING THE EARLY STAGES OF  
*CLOSTRIDIUM DIFFICILE* SPORE GERMINATION

A Dissertation

by

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## ABSTRACT

Infections caused by *Clostridium difficile* have increased steadily over the past several years. While studies on *C. difficile* virulence and physiology have been hindered, in the past, by lack of genetic approaches and suitable animal models, newly developed technologies and animal models allow for improved experimental detail. One such advance was the generation of a mouse-model of *C. difficile* infection. This system was an important step forward in the analysis of the genetic requirements for colonization and infection. Equally important is understanding the differences that exist between mice and humans. One of these differences is the natural bile acid composition. Bile acid-mediated spore germination, a process whereby a dormant spore returns to active, vegetative growth, is an important step during *C. difficile* colonization. Mice produce several different bile acids that are not found in humans (muricholic acids) that have the potential to impact *C. difficile* spore germination. In order to understand potential effects of these different bile acids on *C. difficile* physiology, we characterized their effects on *C. difficile* spore germination and growth of vegetative cells. We found that the mouse-derived muricholic acids affect germination similarly to a previously-described inhibitor of germination, chenodeoxycholic acid.

Chenodeoxycholic acid was previously demonstrated to be a competitive inhibitor of *C. difficile* spore germination, though with what the inhibitors or activators of germination interacted was unknown. However, the inhibitory

property of chenodeoxycholic acid was used in a screen to identify potential germinant receptors and led to the identification of the germination-specific, pseudoprotease, CspC, as the bile acid germinant receptor.

Based on the hypothesized location of CspC within the *C. difficile* spore (cortex rather than inner membrane), we hypothesized that there may be differences between the order of the stages of *C. difficile* and *Bacillus subtilis* spore germination. Germination in *B. subtilis*, a well-studied spore-former, is divided into two genetically separable stages. Stage I is characterized by the release of dipicolinic acid (DPA) from the spore core. Stage II is characterized by cortex degradation, and stage II can be activated by the DPA released during stage I. Thus, DPA release precedes cortex degradation during *B. subtilis* spore germination. To understand how the different location of the *C. difficile* germinant receptor affects the order of DPA release and cortex degradation, we first investigated the timing of DPA release and cortex degradation during *C. difficile* spore germination and found that cortex degradation precedes DPA release. Based on this result and work with SpoVAC in *B. subtilis*, we then investigated germination under high osmolyte concentrations. Because both cortex degradation and DPA release during *C. difficile* spore germination are dependent on the presence of the germinant receptor and cortex degradation, the release of DPA from the core may rely on the swelling of the core upon cortex degradation.

## DEDICATION

This dissertation is dedicated to my mother, Virginia Francis, who always pushed me to strive for more.

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### **Contributors**

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Assistance with experiments in Chapter II was provided by Charlotte A. Allen, who also generated *C. difficile* strain CAA5 used in Chapter III. Work in all chapters was performed under the direction of Dr. Sorg.

All other work described in this thesis was completed by the student, independently.

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## NOMENCLATURE

AGFK	L-asparagine, glucose, fructose and K <sup>+</sup> ions
AMA	$\alpha$ -muricholic acid
BHIS	brain heart infusion supplemented with 5 g / L yeast extract and 0.1% L-cysteine
BMA	$\beta$ -muricholic acid
CA	cholic acid
CaDPA	1:1 chelate of DPA with calcium
CDCA	chenodeoxycholic acid
CDI	<i>Clostridium difficile</i> Infection
CROP	combined repetitive oligopeptide repeat
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
DPA	dipicolinic acid
FC	5-fluorocytosine
FZD	Wnt receptor frizzled family
GCW	germ cell wall
GTD	glucosyltransferase domain
K <sub>i</sub>	apparent inhibitory constant
K <sub>m</sub>	apparent rate of germination
LB medium	Luria-Bertani medium



LCA	lithocholic acid
NAG	N-acetylglycosamine
NAM	N-acetylmuramic acid
OD <sub>600</sub>	optical density at 600nm
OMA	ω-muricholic acid
PG	peptidoglycan
PGA	3-phosphoglyceric acid
PYG media	peptone yeast glucose medium
RNA	ribonucleic acid
SASPs	small acid soluble proteins
SCLEs	spore cortex lytic enzymes
sirA	sporulation inhibitor of replication A
SNPs	single-nucleotide polymorphism
TA	taurocholic acid
TBS	Tris-buffered saline
TBST	TBS supplemented with 1% (vol / vol) Tween*20
TcdA	<i>Clostridium difficile</i> Toxin A
TcdB	<i>Clostridium difficile</i> Toxin B
UDP-glucose	uridine diphosphate glucose
UV	Ultraviolet light
WT	wild-type

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## CHAPTER I

### INTRODUCTION TO SPORE FORMATION IN *BACILLUS SUBTILIS* AND *CLOSTRIDIODES DIFFICILE* AND GERMINATION IN *BACILLUS SUBTILIS*, *CLOSTRIDIUM PERFRINGENS* AND *CLOSTRIDIODES DIFFICILE*

#### **Introduction**

*Clostridioides difficile* (formerly *Clostridium difficile*) is a pathogenic bacterium from Phylum Firmicutes in the Family *Peptostreptococcaceae*, based on 16S rRNA sequencing [1]. *Clostridioides* are Gram-positive, strictly anaerobic bacilli that commonly are motile and produce spores [1]. In PYG media, cells produce straight- and branched-chain saturated and unsaturated fatty acids. During growth, *C. difficile* cells produce chains of two to six cells in an end-to-end alignment. During nutrient limitation, *C. difficile* vegetative cells undergo a developmental program that results in the formation of dormant endospores. Spores are ovular in shape and swell the cell when they form, resulting in the characteristic 'club form' of the cell [1-3].

Originally identified in 1935, *C. difficile* was found in the colonic microflora of healthy, newborn infants [4]. The difficulty presented in culturing the bacterium led to the organism originally being named *Bacillus difficile*. In 1978, Onderdonk and colleagues demonstrated that *C. difficile* causes disease (pseudomembranous colitis) in antibiotic-treated hamsters and that it could be isolated from the diseased animals, thus fulfilling Koch's postulates [5]. Importantly, this work demonstrated that the application of antibiotics,



specifically clindamycin, was necessary to render the host susceptible to infection [5]. Due to the anaerobic nature of the colonic environment, *C. difficile* can colonize and cause disease after the bacterial diversity has been altered through the application of broad-spectrum antibiotics [6]. The normal colonic microbiome is known to provide 'colonization resistance' against invading pathogens, though the mechanisms of this protection are unknown [6].

*C. difficile* infection (CDI) most-commonly presents as cramping and diarrhea. Severe cases of CDI may result in pseudomembranous colitis, toxic megacolon and/or death [5, 7]. The symptoms of CDI are associated with the production and secretion of two toxins, TcdA (toxin A) and TcdB (toxin B) [7-13]. It is hypothesized that toxin B is responsible for clinical symptoms of disease in humans because strains encoding only toxin A are rarely identified in human clinical samples [14, 15]. The toxins are cytotoxic and composed of four different structural domains: the glucosyltransferase domain (GTD), the cysteine protease domain, the translocation domain and the receptor binding domain [a combined repetitive oligopeptide repeat (CROP) domain] [16]. TcdA and TcdB toxins are encoded by separate genes and share both sequence (66%) and structural similarity. The mechanism of action for both toxins is similar [13]. The CROP domain binds to receptors on the target cell (TcdA binds to carbohydrates on the apical surface of epithelial cells while TcdB binds to poliovirus receptor-like 3 and Wnt receptor frizzled family (FZDs) on colonic epithelial cells) which triggers endocytosis [17]. In the endosome, the pH decreases during maturation,

triggering the insertion of the translocation domain, giving the GTD access to the host cytosol. Using inositol hexakisphosphate as a cofactor, the cysteine protease domain cleaves the GTD from the rest of the protein. Using UDP-glucose, the GTD glucosylates Rho-family of GTPases which inactivates them [11-13, 18]. Inactivation of the Rho-family of GTPases leads to cell rounding, loss of colonic barrier function and eventual cell death [13].

Though *C. difficile* growth occurs in the large intestine, exiting the body during the resulting diarrhea is fatal to the strictly anaerobic vegetative cells due to the presence of oxygen. Thus, to transit between hosts, *C. difficile* must survive the exposure to oxygen and to do this, *C. difficile* vegetative cells produce endospores. Endospores are dormant forms of bacteria that, in addition to being oxygen resistant, are resistant to UV radiation, high temperature (>60 °C), desiccation, lysozyme activity, and detergents [19-22].

### **Spore formation**

Endospores are formed in response to a developmental program whereby a vegetative cell places a septum that asymmetrically divides the cell into two compartments. To ensure that this process does not occur during log-phase growth, when nutrients are plentiful, the master transcriptional regulator of spore formation (Spo0A) in all studied endospore-forming organisms must be phosphorylated for its activity [23, 24]. Transcription of Spo0A is under the control of sigma H ( $\sigma^H$ ), a post-exponential growth phase, alternative sigma factor that stimulates production of early sporulation proteins, including KinA and

Spo0E [25, 26]. In *Bacillus subtilis*, a phosphorelay signaling pathway controls the activation of Spo0A by phosphorylation (Figure 1) [23]. Embedded in the plasma membrane are kinases that lead to the activation of Spo0A. Either KinA or KinB phosphorylate Spo0F (Spo0F~P) [26, 27]. Spo0F~P transfers the phosphate to Spo0B and Spo0B~P then transfers the phosphate to Spo0A. There are reports that Spo0A is directly phosphorylated by KinC, but this has only been reproduced in mutants lacking both KinA and KinB and containing point mutations in Spo0A [26, 27]. Spo0E and KinD act to inhibit Spo0A phosphorylation. Interestingly, the abundance of Spo0A~P in the cell leads to differences in transcriptional activity. At low Spo0A~P levels, biofilm formation is activated. However, as Spo0A~P levels increase, *sirA* (sporulation inhibitor of replication A) transcription is initiated [28]. SirA binds directly to the origin of replication, displacing the replication initiation factor DnaA, preventing the cell from undergoing further replication events [26]. At this point, SpoIIIE is produced, which drives, through an unknown mechanism, the relocation of FtsZ to a polar septum [29]. The polar septum divides the cell into two unequally sized compartments: the larger will eventually become the mother cell and the smaller will become the forespore. Following asymmetric division, SpoIIIE then dephosphorylates SpoIIAA [26, 30]. Dephosphorylated SpoIIAA (an anti-anti-sigma factor) binds and sequesters SpoIIAB (an anti-sigma factor), relieving inhibition of SigF ( $\sigma^F$ ), the early forespore-specific RNA polymerase sigma factor [26, 30]. After activation,  $\sigma^F$  recruits RNA polymerase to the *spoIIIR* promoter to

promote expression of the *spoIIIR* gene [26, 31]. SpoIIIR is then secreted into the intermembrane space of the septum separating the mother cell and forespore, where it activates a protease, SpoIIIGA [26]. Activated SpoIIIGA cleaves the first mother cell sporulation-specific RNA polymerase sigma factor, SigE ( $\sigma^E$ ), from pro- $\sigma^E$  to its active form [26].

In a phagocytic-like event, proteins whose expression is controlled by  $\sigma^E$ , initiate forespore engulfment by the mother cell. Though there are several proteins involved in the engulfment process, SpoIIIAH and SpoIIQ (both under  $\sigma^E$  control) are important components. These two proteins interact and act, in a “ratchet-like” fashion, to draw the mother cell membrane over the forming endospore [32]. Forward movement around the forespore is due to random thermal motion but these two proteins prohibit reversal during engulfment. Upon the completion of engulfment, levels of SpoIIQ and SpoIIIAA-AH increase, forming a bridge between the mother cell and the forespore compartments (“feeding tube”) [33, 34]. Subsequently,  $\sigma^F$  activates  $\sigma^G$  expression within the forespore [34]. This potentially occurs through a metabolic limitation that presents in the newly-engulfed forespore [34-36].  $\sigma^G$  activity leads to the expression of late-stage sporulation proteins within the endospore and the activation of  $\sigma^K$  within the mother cell [26, 37, 38].  $\sigma^G$  is required to drive the expression of the proteins necessary for packaging dipicolinic acid (DPA), small acid-soluble protein expression and otherwise preparing the forespore for dormancy.  $\sigma^K$  activity is required to drive the expression of the proteins

necessary for formation of cortex peptidoglycan, coat proteins and, in some spore-forming bacteria, the exosporium [26].

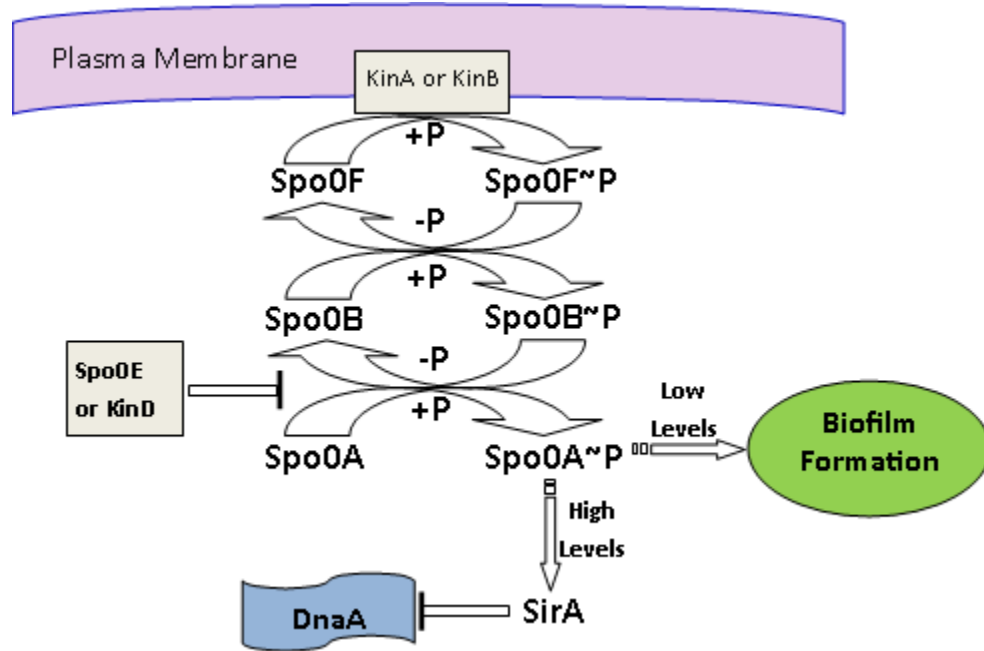


Figure 1. Phosphorylation of Spo0A.

Spo0A is phosphorylated through a cascade beginning with the phosphorylation of Spo0F by either KinA or KinB. Spo0F~P then phosphorylates Spo0B which phosphorylates Spo0A. Either Spo0E or KinD can inhibit the phosphorylation of Spo0A. At low levels, Spo0A drives biofilm formation; at high levels it initiates SirA transcription, which inhibits DNA replication.

Formation of the spore coat depends upon SpoVM. SpoVM detects the difference between the outer forespore membrane and the inner mother cell membrane by detecting the difference in curvature [39, 40]. Once attached to

the membrane, SpoVM functions as an anchor for SpoIVA [41]. SpoIVA hydrolyzes ATP to drive self-assembly and functions as a scaffold on to which the mother cell-derived coat proteins are deposited [39, 42]. One coat protein, CmpA, has been shown to prevent cortex peptidoglycan assembly. SpoVM and SpoIVA repress CmpA, so as spore coat assembly proteins reach the highest concentrations, cortex assembly is derepressed [26].

Cortex formation begins with peptidoglycan (PG) synthesis, an important component of both vegetative cells and spores [43]. PG gives a vegetative cell its strength but is not responsible for the shape of the cell [26]. Cell wall PG is found in both the vegetative cell and the germ cell wall (GCW) of the endospore. The GCW is the precursor of what will become the cell wall of the vegetative cell [26]. Cell wall PG is composed of repeating subunits in an alternating configuration of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues. The stem peptides are attached to the lactyl groups of the NAM residues via their N-terminal linkage and are, generally composed of (Nterm)-L-alanine – D-glutamate – meso-diaminopimelate – D-alanine – D-alanine- (Cterm). The terminal D-alanine is cleaved during crosslinking of the 4th amino acid (D-alanine) to the meso-diaminopimelate of a neighboring glycan strand [26].

Though it is also composed of peptidoglycan subunits, cortex PG is unique. Approximately 50% of the NAM residues found in the cortex PG have had their stem peptide removed which results in a decreased amount of

crosslinks and allows for the formation of muramic- $\delta$ -lactam residues [44]. Originally, this reduced level of crosslinking was hypothesized to allow the cortex to constrict and expand around the core of the spore, granting increased resistance. However, this hypothesis has been disproven. When the amount of cortex crosslinking is artificially increased to the level found in the GCW, there are no effects on the spore's resistance properties [26]. The ability to form muramic- $\delta$ -lactam residues has also been removed, and while this did not have a measurable effect on any resistance properties, it did result in spores with a  $>10^4$  decrease in the ability to form viable colonies [45, 46]. The muramic- $\delta$ -lactam residues are the targets of spore cortex lytic enzymes (SCLEs), allowing the germinating spore to accurately target the cortex for degradation without degrading the GCW PG [45, 47]. Thus, the decrease in colony forming units observed in mutant (above) was presumably due to the inability of the spore to efficiently degrade cortex. Though the cortex is critical for dormancy, wet heat protection and protection against desiccation, there are no protective properties currently associated with the GCW [26, 48].

Located underneath the cortex and GCW is the inner spore membrane where, in most spore-forming bacteria, many germination specific proteins are located (e.g. Ger-type, germinant receptors and proteins important for the packaging and release of DPA, see below). Though the inner membrane is a lipid bilayer, it is relatively impermeable when compared to the plasma membrane of vegetative cells [43]. One hypothesis for this lower permeability is

that the membrane is compressed by the cortex, and that cortex degradation permits the membrane to expand, thereby relaxing permeability. Also, the inner membrane is not nearly as fluid as what is found in vegetative cells, possibly due to the low water content of the spore's core [49].

The spore core is at the center of the spore and is where the genomic DNA, RNA, ribosomes and many enzymes are located. Whereas the vegetative cell is ~88% water, the core has low water content, with water only accounting for ~28-57% of the wet weight [49]. Enriched within the core (~5 – 20% of the dry weight) is DPA and, mostly, is present as a 1:1 chelate with  $\text{Ca}^{2+}$ . DPA is an important spore component which provides the heat resistance commonly associated with spore dormancy and a small amount of resistance against ionizing radiation [48]. In *B. subtilis*, DPA synthesis is controlled by the *spoVF* divergon (composed of the divergently-transcribed *spoVFAB*, *asd*, *dapG* and *dapA* operons) [49, 50]. *spoVFAB* expression is under  $\sigma^K$  control, thus DPA is synthesized in the mother cell during later stages of spore formation [51]. DPA is then packaged into the forespore by the forespore-encoded, DPA-binding protein, SpoVAD. By an unknown mechanism, SpoVAD packages DPA using the SpoVAC channel, rather than the feeding tube (SpoVAC-defective spores are severely DPA deficient) [52-54]. SpoVAA, SpoVAB and SpoVAEb, are also essential for DPA packaging and spore maturation, though their exact roles are not determined [55].



Protecting the DNA against UV radiation and H<sub>2</sub>O<sub>2</sub> are small acid soluble proteins (SASPs) that make up between 5 – 15% of the protein content of the dormant spore [26].  $\alpha$ ,  $\beta$  and  $\gamma$  SASPs are encoded by *ssp* genes (*sspA*, *sspB* and *sspE* respectively) [56]. The  $\alpha$  and  $\beta$  SASPs confer resistance to toxic chemicals and UV radiation [57, 58]. Their binding to DNA also helps to prevent transcription during dormancy. The third SASP,  $\gamma$ , does not appear to have DNA binding capability or confer any resistance properties [56]. During germination, the SASPs are degraded by Gpr, an aspartic acid protease. The degradation of the SASPs free the DNA to allow for transcription to resume and to provide a pool of amino acids for protein synthesis [26]. Gpr is produced as a zymogen during spore formation and activated through autocleavage just prior to spore dormancy. It is unable to cleave its substrates until core hydration during germination is complete [59].

Interestingly, the mechanisms of spore formation in *C. difficile* exhibit significant departures from the *B. subtilis* model. The signal for initiating spore formation in *C. difficile* is currently unknown. Similar to *B. subtilis*, Spo0A is the master sporulation regulator but there is no phosphorelay system that phosphorylates Spo0A to its active form [60]. Several orphan histidine kinases are able to directly phosphorylate Spo0A but how this process is regulated remains unknown. One trigger of sporulation in *C. difficile* is associated with nutrient availability, and several proteins whose functions are associated with metabolism act as repressors of sporulation[3]. For example, Opp and App are

two peptide transporters that inhibit spore formation by an unknown mechanism [61]. CodY, a transcriptional regulator that responds to the abundance of branched chain amino acids and GTP, also to suppresses sporulation when nutrients are abundant [62]. Inactivation of *opp*, *app* or *codY* increase sporulation frequency [61, 62].

Predivisinal *C. difficile* cells express three sporulation-associated proteins, SpoIIAB, SpoIIAA and SpoIIIE [3, 30, 63]. These proteins appear to be involved in early septum formation at the start of asymmetric division. After the septum formation, the expression of  $\sigma^F$  and  $\sigma^E$  begins in the forespore and mother cell compartments, respectively [64]. In *C. difficile*,  $\sigma^E$  expression is under partial control of  $\sigma^F$  rather than under full control – it can be activated in the absence of  $\sigma^F$  [65]. Independent of both  $\sigma^E$  activity and engulfment,  $\sigma^G$  is activated in the forespore through an unknown post-translational mechanism [3]. In the mother cell,  $\sigma^K$  is expressed in its active form, not in the pro-form observed in other organisms, and *C. difficile* does not encode a SpoIVFB orthologue which processes pro- $\sigma^K$  to the active form [65]. Cortex formation requires  $\sigma^G$  rather than  $\sigma^K$  ( $\sigma^K$ -mutant spores still assemble cortex) [64]. This leads to a sigma factor cascade model that diverges at the beginning of spore formation and proceeds independently of what occurs in the other compartments. The *B. subtilis* model of spore formation depends on crosstalk between the forespore and the mother cell, while *C. difficile* appears to have a significantly reduced level of cross-talk.

## Spore germination

The nature of a metabolically-dormant spore requires that the processes occurring during germination (*i.e.*, degradation of SASPs, DPA release, cortex degradation) occur in an energy independent manner. As a result, the initiation of germination and loss of spore dormancy must be pre-programmed (*i.e.*, enzymatic). *B. subtilis* has been a model for spore germination and most spore-formers have orthologues of germination-related proteins found in *B. subtilis*. Germination in *B. subtilis*, and all studied spore-forming bacteria, begins with a germinant binding to the germinant receptor [66]. Germinants are small molecules, most commonly nutrients, which bind to their cognate germinant receptor to trigger germination. In *B. subtilis*, germination is triggered by either L-alanine or a mixture of L-asparagine, glucose, fructose and K<sup>+</sup> ions (AGFK) [67]. The receptors are encoded by three homologous tricistronic operons, *gerA*, *gerB*, and *gerK* [66, 68, 69]. The GerA receptor (composed of the GerAA, GerAB & GerAC proteins) recognizes L-alanine, while the combined actions of the GerB and GerK (composed of the GerBA, GerBB & GerBC and GerKA, GerKB & GerKC proteins, respectively) receptors recognize AGFK [66, 70].

Following germinant binding, cations are released, followed by release of DPA from the spore core. The release of DPA is associated with the proteins encoded by the *spoVA* operon (*spoVAA*, *spoVAB*, *spoVAC*, *spoVAD*, *spoVAEa*, *spoVAEB*, *spoVAF*) (Figure 2) [54]. The functions of many of these proteins are unknown, however most are essential for DPA packaging or release during

spore formation and germination, respectively [52, 71, 72]. However, SpoVAD was shown to bind to DPA and SpoVAC has mechanosensing properties and is associated with DPA release [53, 54]. SpoVAEa and SpoVAF are non-essential for spore germination. *spoVAEa* and *spoVAF* mutants germinate but germination occurs at a slower rate [55]. The release of cations and DPA from the spore completes Stage I of spore germination [66].

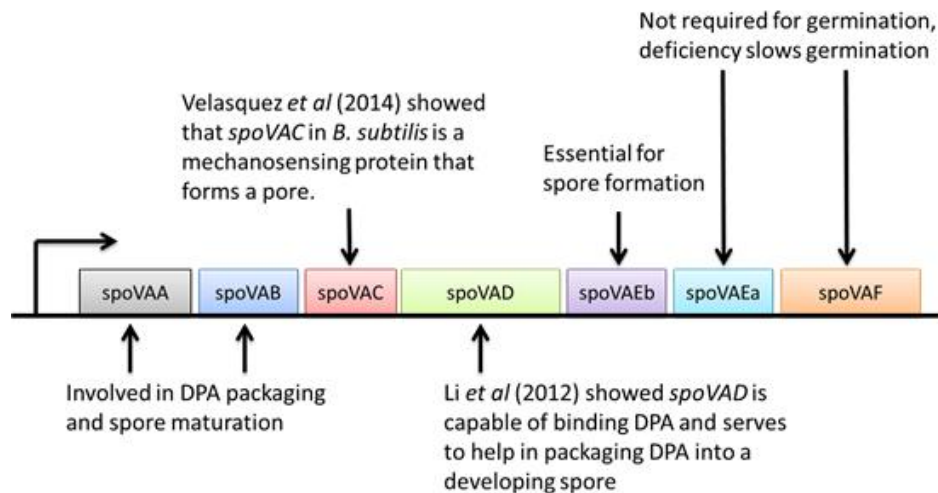


Figure 2. *B. subtilis* *spoVA* operon

In *B. subtilis*, the release of DPA from the core activates Stage II of germination. Stage II consists of cortex degradation, complete core hydration and expansion and loss of the resistance properties normally associated with dormancy (e.g., heat resistance) [66]. In *B. subtilis*, cortex degradation is

controlled by two SCLEs, CwlJ and SleB. Spores can degrade their cortex using either enzyme but germination by *B. subtilis*  $\Delta cwlJ \Delta sleB$  spores cannot proceed past Stage I of germination [73]. Both enzymes target the muramic- $\delta$ -lactam residues of the cortex peptidoglycan, with SleB acting as a lytic transglycosylase [73]. SleB is synthesized in the fore-spore and localizes to the inner spore membrane, just outside the core. Conversely, CwlJ is synthesized in the mother cell and is hypothesized to be localized to the spore coat, though the location of the protein in the spore and its mechanism of action has not been adequately determined [74, 75].

The activities of these SCLEs must be regulated in a way that prevents their activity during dormancy. SleB activity is inhibited by YpeB, which is degraded after germination begins, thereby allowing SleB access to the cortex PG [73]. CwlJ is activated by the DPA that is released from the spore core and exogenous DPA also can trigger CwlJ activity [75]. With the cortex degraded, the core is able to continue to rehydrate and expand. This stage of reactivation and resumption of protein synthesis is termed 'ripening' [66]. The spore has a limited supply of stored ATP, so the energy required for this stage is provided by metabolizing 3-phosphoglyceric acid (PGA). PGA is an acid-soluble spore phosphorous compound that is an intermediate in both the Calvin cycle and glycolysis and functions as a stable energy reservoir for the spore [76].

Spore germination in most of the identified spore-forming bacteria is most similar to what is observed for *B. subtilis* with the major variation being

differences in what germinants activate the process. However, a few identified bacteria form spores and germinate by novel methods, including *Clostridium perfringens* and *C. difficile*. *C. perfringens* is an anaerobic spore forming bacteria that inhabits the soil and intestines of humans and animals, and is the causative agent of clostridial myonecrosis (gas gangrene) and mild enterotoxemia [77].

Germination in *C. perfringens* differs from *B. subtilis* with the products of the *gerK* operon serving as the primary germinant receptors [78]. The operons are organized into two separate transcriptional units, the monocistronic *gerKB* gene and the bicistronic *gerKA-KC* genes [79]. GerKA and GerKC are required for L-asparagine and KCl-mediated germination. Though GerKB is important for germination, it appears to not play a direct role in germinant detection [79]. Rather is required for spore viability and outgrowth. Though *C. perfringens* encodes a potential *gerA* ortholog, it has little effect on germination. In another departure from the mechanisms of germination found in *B. subtilis*, *C. perfringens* does not encode any *B. subtilis* SCLEs orthologues. *C. perfringens* encodes *sleC* and *sleM* [80, 81]. SleC acts on intact spore cortex and is an N-acetylmuramyl-L-alanine amidase [81]. SleM is an N-acetylmuramidase and acts on disrupted peptidoglycan fragments following SleC degradation [80]. Both enzymes are localized outside of the spore cortex layer, but only SleC is essential for germination.

Similar to what is observed in *B. subtilis*, the SCLE activities in *C. perfringens* must be regulated to prevent premature cortex degradation. To prevent such an occurrence, SleC is deposited into the *C. perfringens* spore as a zymogen, pro-SleC [82]. To generate active SleC enzyme, the pro-sequence must be removed, and this occurs early during germination by a group of germination-specific, subtilisin-like, serine proteases – the Csp proteins. Many *C. perfringens* strains encode three Csp proteases, *cspA*, *cspB* and *cspC* [82, 83]. All three of these proteases are capable of cleaving pro-SleC *in vitro*. *C. perfringens* strain SM101 only encodes one active protease, CspB, and is it essential for cortex hydrolysis and rapid DPA release. Thus, this presents a different model of spore germination, when compared *B. subtilis*, wherein DPA release from the core may not directly activate SCLEs. Rather the Csp protein(s) are activated, by an unknown mechanism, to cleave the SCLEs and initiate cortex degradation.

### ***C. difficile* spore formation and germination**

Germination by *C. difficile* spores is initiated by a combination of certain bile acids and amino acids (glycine has highest efficiency in laboratory conditions) [84, 85]. Bile acids are synthesized in liver hepatocytes using cholesterol as a precursor and secreted into the gut to aid in the absorption of fats during digestion. Humans produce two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), while rodents produce other bile acids (muricholic acids, see Chapter II and Figure 3) in greater amounts than CDCA

[86-88]. These primary bile acids are then modified through conjugation with either a taurine [producing taurocholic acid (TA) or taurochenodeoxycholic acid] or glycine (producing glycocholic acid or glycochenodeoxycholic acid) at the C-24 carboxyl group [88]. During digestion, the bile acids aid traffic through the small intestine and most are reabsorbed and recycled to the liver to be used in other rounds of digestion [89]. Though efficient, enterohepatic recirculation is not 100%. Approximately 5 – 10% of the total bile acid pool escapes this process and enters the large intestine where it becomes modified by the resident microbiome [90].

Many members of the colonic microbiome express on their cell surfaces bile salt hydrolases, Bsh, that cleave the conjugated taurine or glycine from the base CA or CDCA structures (deconjugation) [88]. Subsequently, specific bacteria in the microbiome actively take up the deconjugated CA and CDCA and convert them to deoxycholic acid (DCA) and CDCA to lithocholic acid (LCA), respectively [88]. This 7 $\alpha$ -dehydroxylation reaction proceeds to completion in a healthy gut (primary bile acids are not found in a healthy gut – they are metabolized to secondary bile acids). The presence and concentrations of these different bile acids can impact the gut microbiome potentially due to their detergent-like effects. How bile acids induce bacterial cell death is not currently known, but it has been suggested that the presence of bile acids may elicit stress response membrane synthesis and protection or trigger various DNA repair pathways [91-93]. Because bile acids can be potentially toxic to bacteria,



different mechanisms have been developed to tolerate or alter what bile acids are present. Whereas some bacteria modify bile acids (e.g., 7 $\alpha$ -dehydroxylation), many have export mechanisms for expelling bile acids [94].

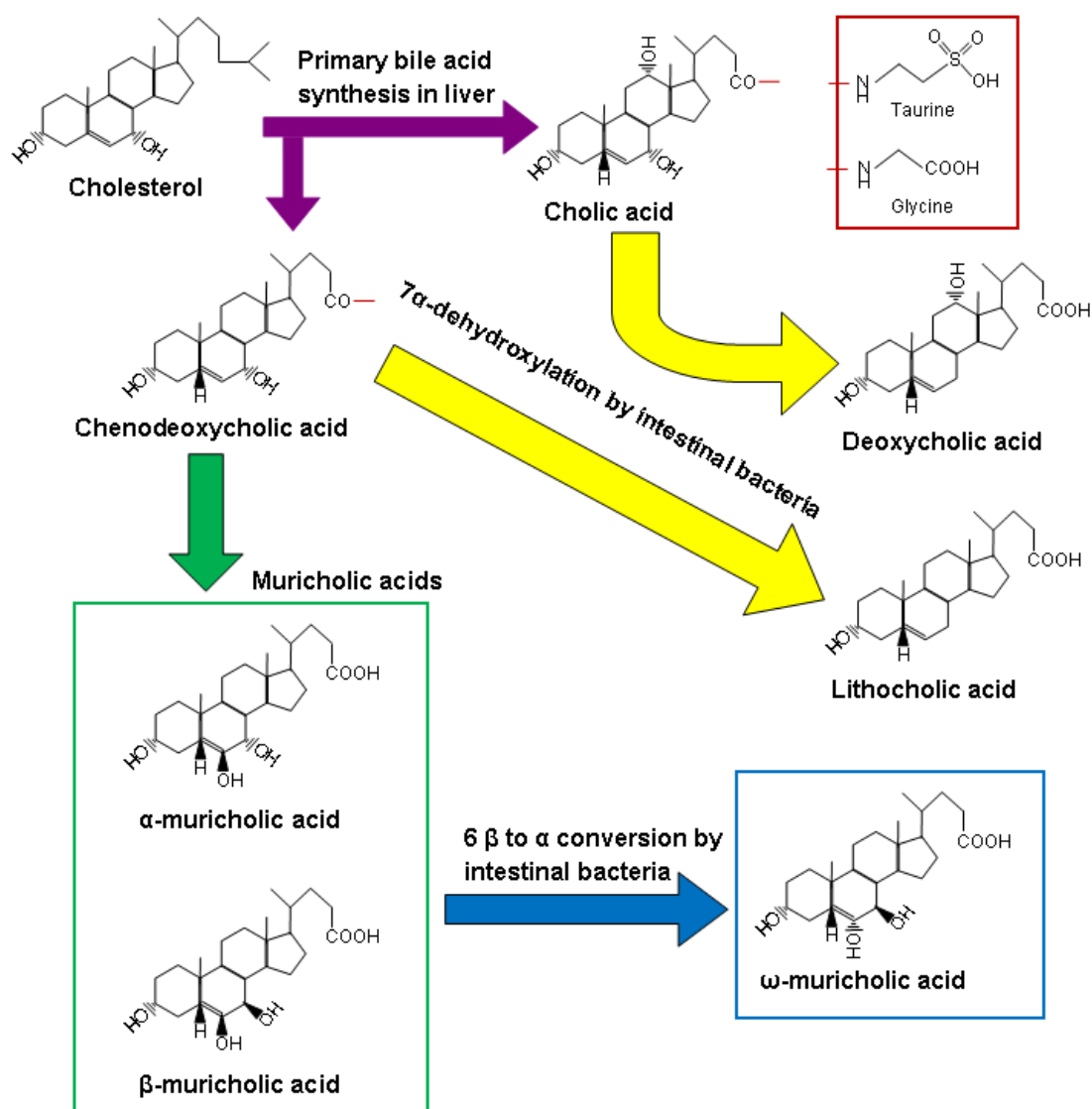


Figure 3. Bile acid synthesis and conversion

For *C. difficile*, CDCA, LCA and DCA are toxic to vegetative cells, though the mechanism of toxicity is unknown. Whereas, *C. difficile* spores recognize CA and its derivatives (including DCA) as germinants, CDCA-derivatives act as inhibitors of CA-mediated germination [84, 85, 95, 96]. In our working model, ingested *C. difficile* spores encounter approximately equal ratios of CA- and CDCA-derivatives in the host's small intestine. Under these conditions, germination would be inhibited because the *C. difficile* spore interacts with CDCA with greater apparent affinity [96]. Thus, germination would be blocked in the aerobic environment of the small intestine. In the anaerobic environment of the colon, however, CDCA is passively absorbed by the colonic epithelium at a ten-times greater rate than CA [89]. Thus, in the antibiotic-treated colon, the ratio of CA to CDCA shifts towards CA and, thus, germination-promoting conditions. Though the signals that stimulate germination are known, with what the germinants interact was originally unknown. This was largely due to the absence of *ger*-type orthologues in the *C. difficile* genome [97].

Using a chemical mutagenesis screen to identify candidate genes, our laboratory demonstrated that a homolog of *C. perfringens* CspC is the *C. difficile* bile acid germinant receptor [98]. Though they are homologous, there are several important differences between the Csp proteins found in *C. difficile* and *C. perfringens*. In *C. difficile*, the *csp* operon that encodes *cspC* also encodes *cspB* and *cspA*. However, unlike what is observed in *C. perfringens*, *C. difficile*

*cspB* and *cspA* are translationally fused and generate CspBA that is then cleaved to CspB and CspA, presumably by the YabG protease [99]. During germination and following the germinant / germinant receptor interaction, the *C. difficile* cortex hydrolase, SleC, must be cleaved from its inactive pro-form by the Csp proteins. Importantly, *C. difficile* CspA and CspC are catalytically inactive pseudoproteases (they lack the catalytic triad necessary for activity), and are unable to cleave the pro-SleC to its active form [99]. However, *C. difficile* CspB contains an active catalytic triad and has the potential to process pro-SleC. In our working model, we hypothesize that CspC binds to the bile acid germinant and, through an unknown mechanism, triggers CspB to cleave pro-SleC into SleC [98]. How and where the amino acid co-germinant binds is currently unknown.

Another important regulator of *C. difficile* spore germination is GerS [100]. GerS is synthesized in the mother cell under  $\sigma^E$  control and appears to localize to the inner leaflet of the outer spore membrane. Here, it is hypothesized to act as an anchor for other germination proteins (e.g., CspA, CspB, CspC and pro-SleC). During germination, *C. difficile*  $\Delta$ *gerS* spores process the germinant signal and cleave pro-SleC into SleC [100]. Though processed to an active form, SleC does not degrade cortex suggesting that GerS may help to release active SleC from the complex. Supporting this idea is recent data showing that along with acting as the germinant receptor, CspC also appears to have an inhibitory effect on germination [101].

The model for *C. difficile* spore germination has presented (Figure 4) several interesting deviations from what is observed for *B. subtilis* spore germination. One of the first departures from the mechanisms of spore germination found in *B. subtilis* (and *C. perfringens*) was that the germinant receptor appears to be located in the cortex [66, 98]. In our working model for *C. difficile* spore germination, we proposed that CspC activates the CspB protease which then cleaves SleC to its active form. This model would suggest that germinant binding could lead to cortex hydrolysis and then DPA release, opposite to what has been observed for *B. subtilis* and all other spore-forming bacteria studied to date. If cortex hydrolysis occurs before DPA release, how is DPA release initiated? In the following chapters, we will discuss how we determine when spore cortex hydrolysis occurs and potentially how DPA is released.

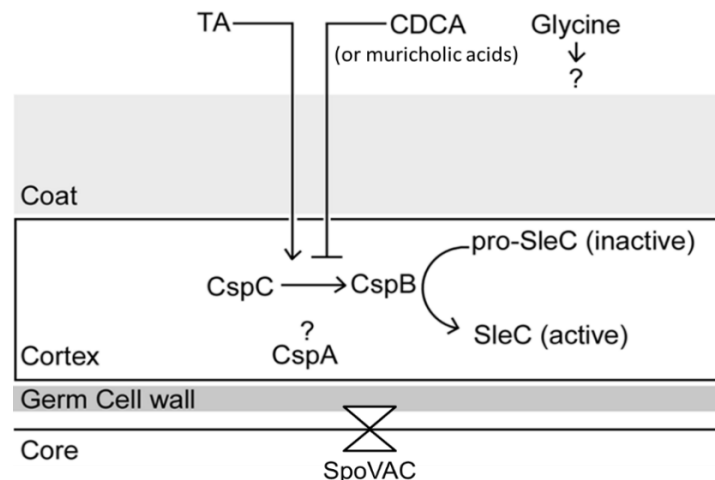


Figure 4. Model for *C. difficile* germination

## CHAPTER II

### MURICHOLIC ACIDS INHIBIT *CLOSTRIDIUM DIFFICILE* SPORE GERMINATION AND GROWTH\*

In Chapter II, we analyze the effects of mouse-derived bile acids (muricholic acids) on the germination of *C. difficile* spores. These bile acids,  $\alpha$ ,  $\beta$  and  $\omega$  muricholic acids, were tested for both their effects on spore germination and vegetative growth using two *C. difficile* strains.

#### Introduction

*Clostridium difficile* is an anaerobic, spore-forming bacteria that is the leading cause of antibiotic-associated diarrhea. As the costs associated with treatment continue to rise [102, 103], much research has focused on understanding the normal course of infection within humans. One of the challenges in the study of *C. difficile* infections has been developing suitable animal models that adequately reproduce symptoms as presented in humans. Gnotobiotic neonatal piglets, rats, and germ-free mice have all been used to varying degrees of success [104-107]. The most widely used model has been the Syrian hamster model of *C. difficile* disease [5, 108-111]. Antibiotic-treated hamsters are very sensitive to *C. difficile* infection with lethal disease presenting approximately 3 days after inoculation by *C. difficile* spores. While the hamster

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represents an excellent model of acute disease, hamsters typically succumb too quickly to disease to measure factors influencing colonization, representing only the full presentation of disease and not less severe symptoms when exposed to epidemic strains [112]. Such rapid progression of the disease and high mortality can also pose problems when attempting to study relapsing infection.

Several mouse models of infection have been developed [106, 113-117]. Some of these models use heavy doses of antibiotics (e.g. kanamycin, gentamicin, colistin, metronidazole and vancomycin followed by clindamycin or cefoperazone followed by clindamycin) and then inoculation with *C. difficile* spores or vegetative cells [114, 115]. These antibiotic regimens sensitize mice so that they respond to infection in a dose dependent manner (increasing disease severity with increasing number of dosed cells or spores). Further, antibiotic-treated mice can relapse after a course of antibiotic treatment, to cure the primary infection, and will express some resistance to reinfection when allowed to fully recover from disease [114]. These are important components of an animal model because relapse in humans represents one of the main challenges to current treatment regimens [118, 119].

Because the mouse model is beginning to be a more widely accepted method of testing potential preventative therapies [120] and the genetic requirements for infection [113, 121, 122], it is important to understand what potential variability exists between the mouse model of infection and humans. One potential source of variability is the natural differences between mouse and

human microbiota. The use of an antibiotic cocktail before infection is an attempt to impact these other microbes [123, 124]. Another important source of variability is the differences in the natural fecal bile acid composition between mice [86, 125] and humans [88] (and hamsters [126-128]).

In humans, bile acids are synthesized in the liver as either cholic acid (3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-5 $\beta$ -cholanic acid) or chenodeoxycholic acid [3 $\alpha$ , 7 $\alpha$ , -dihydroxy-5 $\beta$ -cholanic acid (CDCA)] [88]. These bile acids are then conjugated with either taurine or glycine and, later, further modified by certain members of the colonic microbiota [88]. Previous work has shown that colony formation by *C. difficile* spores on rich medium occurs after exposure to cholic acid derivatives [84, 85]. Subsequent work has shown that all cholic acid derivatives and some amino acids, commonly glycine, can stimulate the initiation of spore germination while CDCA-derivatives are competitive inhibitors of cholic acid-mediated germination [85, 95, 96, 129, 130]. In mice and rats, CDCA is a component of bile, but there are two additional bile acids,  $\alpha$ -muricholic acid (AMA) and  $\beta$ -muricholic acid (BMA), that are not present in humans [89]. A third muricholic acid,  $\omega$ -muricholic acid (OMA), is an epimer of BMA and is produced by the normal microbiota. The effects of these compounds on *C. difficile* spore germination are unknown.

Germination by *C. difficile* spores must be the first step in colonization [98, 120]. The toxins necessary for disease are not found within the spore or deposited on the outer layers during spore formation [131]. To generate active

infection in the hamster model of *C. difficile* disease, approximately 100 spores will result in a lethal infection (LD<sub>100</sub>) while in the mouse model, significantly more spores are required to generate lethal disease ( $\sim 10^8$ ) [120]. Interestingly, when vegetative cells are used to inoculate antibiotic-treated mice, fewer cells are needed ( $\sim 10^5$ ), suggesting the efficiency of *in vivo* germination by *C. difficile* spores may be affected differently in the mouse than in the hamster [114, 120, 132].

Here, we investigate how muricholic acids affect *C. difficile* spore germination and growth using two *C. difficile* isolates; UK1 – an epidemic ribotype 027 isolate [96, 133] and M68 – a ribotype 017 that readily colonizes mice [113, 117]. We find that all three muricholic acids can inhibit *C. difficile* spore germination with apparent affinities similar to what is observed for CDCA and that these compounds are also growth inhibitory.

## **Materials and methods**

### *C. difficile* growth conditions

*C. difficile* strain UK1 [96, 98, 133, 134] and strain M68 [113, 135] were grown in BHIS medium (Brain Heart Infusion supplemented with 5 g / L yeast extract and 0.1% L-cysteine) at 37 °C in an anaerobic environment (85% nitrogen, 10% hydrogen and 5% carbon dioxide).

### *C. difficile* spore preparations

Spores of *C. difficile* UK1 and *C. difficile* M68 were prepared as described previously [96, 134, 135]. Briefly, *C. difficile* UK1 or M68 were streaked on BHIS



agar medium and incubated for 4 days under anaerobic conditions at 37 °C. Plates were then removed from the chamber and cell matter was scraped and diluted into 1 mL of water. Tubes were then left to incubate overnight at 4 °C to aid in the release of spores from the mother cell. The next day, cell matter was resuspended and centrifuged at 14,000 x g for 1 minute. Tubes were decanted and resuspended in 1 mL of water. After 5 washes, the pellets from several tubes were combined in 2 mL water and layered on top of 8 mL of 50% sucrose. Spores were separated from vegetative cells and cell debris by centrifugation for 20 minutes at 4,000 x g. All liquid was then removed from the tube. The pellet, containing the purified spores, was resuspended in 1 mL of water. The purified spores were washed in water as described above. When examined by phase-contrast microscopy, the remaining pellet appeared to be composed >99.9% phase-bright spores.

#### Germination of *C. difficile* spores

Purified spores were heat activated for 30 min at 65 °C and placed on ice, as described previously [95, 96, 134, 136, 137]. Heat-activated spores were then diluted into 990 µL BHIS supplemented with 0 mM, 2 mM, 5 mM, 10 mM, 20 mM or 50 mM taurocholate. When testing muricholic acids or CDCA, bile compound was added to tubes before the addition of spores. The initiation of germination was followed by monitoring absorbance at 600nm. The ratio of the  $A_{600}$  at time X ( $T_x$ ) to the  $A_{600}$  at time zero ( $T_0$ ) was plotted against time. Germination rates, and apparent affinities, were determined using the slopes of

the linear portions of the germination plots, as described previously [96, 134, 137]. Data are reported as the averages from three independent experiments with one standard deviation from the mean. For clarity, only every fourth data point is plotted. CDCA, AMA, BMA and OMA were dissolved at 100 mM in 100% ethanol. AMA, BMA and OMA were purchased from Steraloids, Inc (Newport, RI).

#### Minimum inhibitory concentration

*C. difficile*, from an actively growing plate, was grown overnight in 5 mL liquid BHIS under anaerobic conditions. The next day, 25 mL BHIS medium was inoculated with 0.25 mL of the overnight *C. difficile* culture and then incubated until an OD<sub>600</sub> of 0.45. One hundred twenty five-microliters of this culture then added to 50 mL of ice cold reduced BHIS and kept on ice. Microtiter plates containing BHIS and serially diluted compound were previously prepared and placed in anaerobic chamber to reduce. 10 µL of chilled cells were then added to wells and incubated for 24 hours at 37°C. After 24 hours, plates were removed from the anaerobic chamber and growth measured using a BioRad Xmark plate reader.

#### Statistical significance

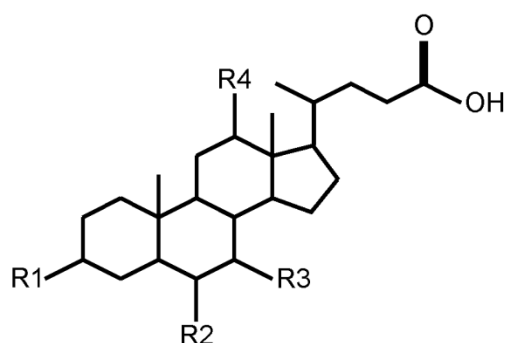
Experiments were performed in triplicate and data represent the average of the three independent experiments. Statistical significance between UK1 and M68 was determined using the Student's T-test.

## Results

### Structures of muricholic acids

Mice synthesize three bile acids not found in humans. Two of these compounds are synthesized directly by the mouse; AMA (3 $\alpha$ , 6 $\beta$ , 7 $\alpha$ -trihydroxy-5 $\beta$ -cholanolic acid) and BMA (3 $\alpha$ , 6 $\beta$ , 7 $\beta$ -trihydroxy-5 $\beta$ -cholanolic acid) (Figure 5) [86, 125]. The third muricholic acid, OMA (3 $\alpha$ , 6 $\alpha$ , 7 $\beta$ -trihydroxy-5 $\beta$ -cholanolic acid) is produced by oxidation of the 6 $\beta$ -hydroxyl of  $\beta$ -muricholic acid followed by reduction of the compound to a 6 $\alpha$ -hydroxyl group (Figure 5) by members of the mouse colonic microbiota [138, 139].

AMA and BMA contain a 6 $\beta$ -hydroxyl group while OMA contains a 6 $\alpha$ -hydroxyl group (Figure 5). The conformational effect of this 6-hydroxyl group is untested on *C. difficile* spore germination because bile acids normally found in the human gut lack the 6-hydroxyl group. As shown in Figure 5, all three muricholic acids lack a 12 $\alpha$ -hydroxyl group, suggesting they might act as inhibitors of *C. difficile* spore germination [96].



Bile Acid	R - group			
	R1	R2	R3	R4
Cholic Acid	$\alpha$ OH	-	$\alpha$ OH	$\alpha$ OH
Deoxycholic Acid	$\alpha$ OH	-	-	$\alpha$ OH
Chenodeoxycholic Acid	$\alpha$ OH	-	$\alpha$ OH	-
Lithocholic Acid	$\alpha$ OH	-	-	-
$\alpha$ - Muricholic Acid	$\alpha$ OH	$\beta$ OH	$\alpha$ OH	-
$\beta$ - Muricholic Acid	$\alpha$ OH	$\beta$ OH	$\beta$ OH	-
$\omega$ - Muricholic Acid	$\alpha$ OH	$\alpha$ OH	$\beta$ OH	-

Figure 5. Structures of common muricholic acids.

The primary bile acids (cholic acid, chenodeoxycholic acid,  $\alpha$ -muricholic acid and  $\beta$ -muricholic acid) are listed. Deoxycholic acid and  $\omega$ -muricholic acid are secondary bile acids and are products of the normal microbiota.

#### Muricholic acids inhibit *C. difficile* spore germination

To understand how these compounds affect germination, *C. difficile* spores were assayed for germination in the presence or absence of muricholic acids. As positive and negative controls, respectively, the initiation of spore germination was followed in the presence of taurocholic acid, a known *C. difficile* spore germinant [84, 85] or in the presence of taurocholic acid and CDCA, a known inhibitor of *C. difficile* spore germination [95, 96].

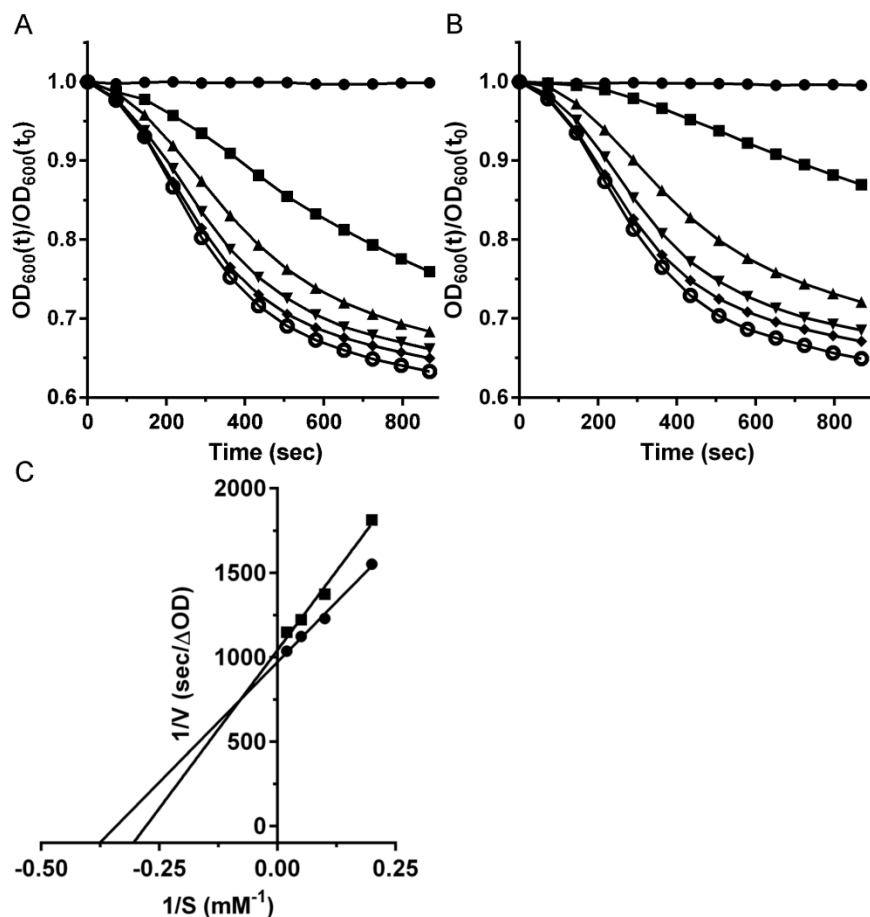


Figure 6.  $\alpha$ -muricholic acid inhibits germination by *C. difficile* UK1 spores. (A) Germination of *Clostridium difficile* UK1 spores in complex medium supplemented with taurocholic acid (TA) or (B) medium supplemented with TA and 1 mM CDCA or (C) medium supplemented with TA and 1 mM  $\alpha$ -muricholic acid.  $\bullet$  0 mM TA,  $\blacksquare$  2 mM TA,  $\blacktriangle$  5 mM TA,  $\blacktriangledown$  10 mM TA,  $\blacklozenge$  20 mM TA or  $\circ$  50 mM TA. (D) The inverse rate ( $1/v$  [sec/ $\Delta OD$ ]), versus the inverse taurocholate concentration ( $1/S$  [ $mM^{-1}$ ]), was plotted. Apparent  $K_m$  values for TA alone ( $\bullet$ ) and in the presence of  $\alpha$ -muricholic acid ( $\blacksquare$ ) were determined from the linear best fit.

Purified *C. difficile* UK1 spores were suspended in BHIS medium and different taurocholic acid concentrations (Figure 6A). As described previously, the rate of germination increased with increasing taurocholic acid concentration [96, 134, 137]. The addition of 1 mM CDCA had an inhibitory effect on germination (Figure 6B). The addition of 1 mM AMA resulted in a clear reduction of the ability of *C. difficile* spores to germinate in response to TA (Figure 6C). The effect of this inhibition of germination was quantified by applying Michaelis-Menten kinetics to the germination plots to generate apparent  $K_m$  values. While not traditional enzyme kinetics, these types of analyses have aided in the identification of the requirements for spore germination and for novel inhibitors of spore germination [96, 130, 134, 136, 137, 140, 141]. Analysis of the Lineweaver-Burk plot of *C. difficile* UK1 spore germination in taurocholic acid alone (Figure 6D) yielded an apparent  $K_m$  value similar to what has been previously reported (Table 1) [96, 134]. When analyzing germination by *C. difficile* UK1 spores in the presence of different muricholic acids, it was immediately obvious that these compounds were germination-inhibitory. From the germination plots, we determined the rates of germination and used this data to generate apparent inhibitory constants ( $K_i$ ) for each inhibitor tested (Table 1). Comparing the muricholic acids to CDCA, BMA and OMA yielded apparent inhibition constants similar to CDCA while AMA proved to be least efficient at inhibiting germination (Table 1). The difference between CDCA and AMA can be observed by comparing the ability of *C. difficile* spores to germinate in BHIS

medium supplemented with 2 mM TA. Addition of 1 mM CDCA had a greater effect than did the addition of 1 mM AMA.

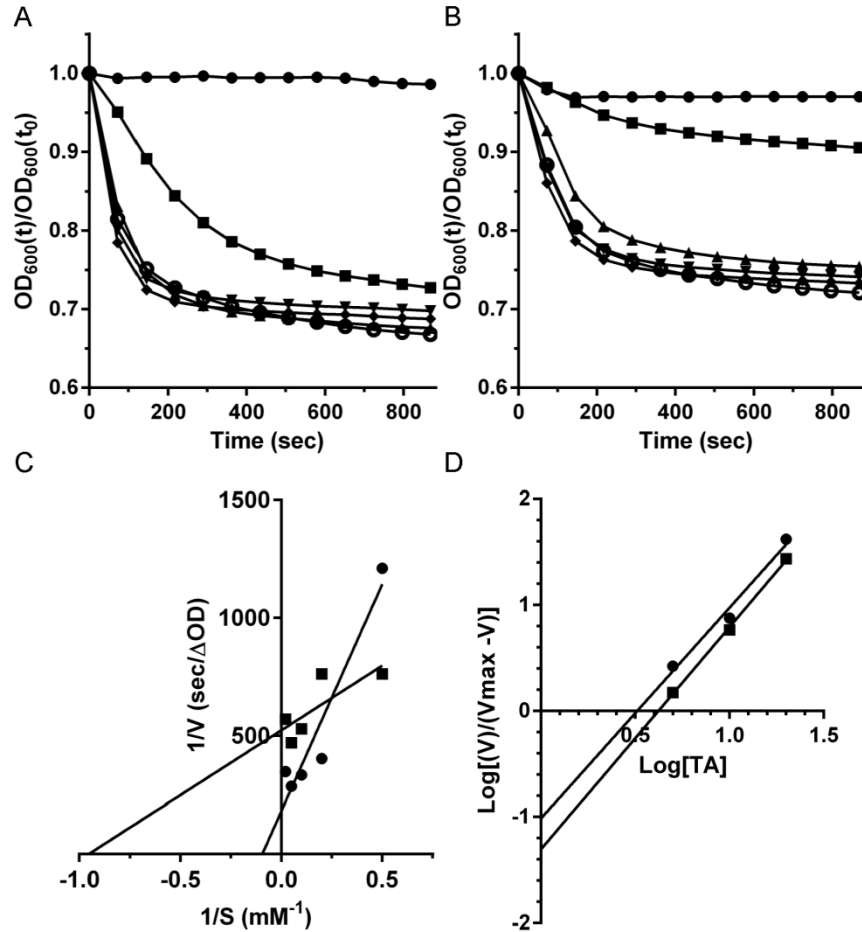


Figure 7.  $\alpha$ -muricholic acid inhibits germination by *C. difficile* M68 spores. (A) Germination of *Clostridium difficile* M68 spores in complex medium supplemented with taurocholic acid (TA) or (B) medium supplemented with TA and 1 mM  $\alpha$ -muricholic acid. ● 0 mM TA, ■ 2 mM TA, ▲ 5 mM TA, ▼ 10 mM TA, ◆ 20 mM TA or ○ 50 mM TA. (C) The inverse rate ( $1/v$  [sec/ $OD_{600}$ ]), versus the inverse taurocholate concentration ( $1/S$  [ $mM^{-1}$ ]), was plotted. Apparent  $K_m$  values for TA alone (●) and in the presence of  $\alpha$ -muricholic acid (■) were determined from the linear best fit of the plotted data. (D) Hill Plot was generated to determine the apparent  $K_m$  values for each condition.

Germination of *C. difficile* M68 in medium with taurocholic acid was similar to *C. difficile* UK1. *C. difficile* M68 rapidly germinated in medium supplemented with taurocholic acid (Figure 7A) and was inhibited when 1 mM AMA was added to the germination solution (Figure 7B). However, by analyzing the kinetics of *C. difficile* M68 spore germination, we observed that the data from this strain produced non-linear Lineweaver-Burk plots (Figure 7C), a phenomenon observed for some other *C. difficile* strains [137]. The Hill plot (Figure 7D) was used to generate the apparent  $K_m$ . This value was then used to determine the apparent  $K_i$  under each condition tested. When *C. difficile* M68 spores were germinated in the presence of CDCA, germination was strongly inhibited (Table 1). The inhibition of germination by BMA was similar to the inhibition observed for *C. difficile* UK1 and, again, AMA was the least efficient at inhibiting spore germination (Table 1). OMA was a more potent inhibitor of *C. difficile* M68 spore germination than *C. difficile* UK1 spore germination (p-value < 0.05).



Table 1. Bile acid effects on *C. difficile* spore germination

Strain	UK1	M68
	$K_m$ (mM)	$K_m$ (mM)
Taurocholic Acid	$3.2 \pm 0.5$	$3.5 \pm 0.5$
	$K_i$ (mM)	$K_i$ (mM)
Chenodeoxycholic Acid	$0.22 \pm 0.07$	$0.12 \pm 0.02$
$\alpha$ -Muricholic Acid	$0.62 \pm 0.09$	$0.59 \pm 0.05$
$\beta$ -Muricholic Acid	$0.27 \pm 0.12$	$0.26 \pm 0.02$
$\omega$ -Muricholic Acid	$0.29 \pm 0.03$	$0.20 \pm 0.01^*$

$$K_i = [\text{inhibitor}] / [(K_{m,TA} \text{ with inhibitor}) / ((K_{m,TA} \text{ without inhibitor}) - 1)]$$

\*  $p < 0.05$

#### Minimum inhibitory concentration of muricholic bile acids

Previously, we demonstrated that CDCA and deoxycholic acid inhibited *C. difficile* growth [85]. In antibiotic-treated mice, the levels of deoxycholic acid are likely to be very low because it is a product of the  $7\alpha$ -dehydroxylation of cholic acid by the normal microbiota [88]. However, cholic acid, CDCA, AMA and BMA will be present and could affect *C. difficile* growth. To quantify the effects of these compounds on *C. difficile* growth, we determined the MIC. Serial, 2-fold dilutions of bile acids in growth media were used to determine the MIC for each bile acid (Table 2). *C. difficile* strain UK1 did not grow in the presence of CDCA or AMA or BMA, at a concentration of 1 mM or above. OMA was less toxic to the strain; a concentration of 2 mM was necessary to inhibit growth. In stark contrast, the MIC of cholic acid for *C. difficile* UK1 was 10 mM,

a concentration not found in the colon. We observed slightly different results when analyzing the MIC of these bile acids for *C. difficile* M68 growth. This strain was more resistant to the toxic effects of AMA and BMA while equally as sensitive to CDCA, cholic acid and deoxycholic acid (Table 2). BMA is more prevalent in the gut of rats and mice than is CDCA [125], suggesting that a strain which is more resistant to the toxic effects of BMA (e.g. *C. difficile* M68) might be able to better colonize mice.

Table 2. Minimum inhibitory concentration of bile acids for *C. difficile* strains

Strain	UK1 (mM)	M68 (mM)
Cholic Acid	10.0 ± 0.0	10.0 ± 0.0
Deoxycholic Acid	1.0 ± 0.0	1.0 ± 0.0
Chenodeoxycholic Acid	1.0 ± 0.0	1.0 ± 0.0
α-Muricholic Acid*	1.0 ± 0.0	2.0 ± 0.0
β-Muricholic Acid*	1.0 ± 0.0	2.0 ± 0.0
ω-Muricholic Acid	2.0 ± 0.0	2.0 ± 0.0

The MIC did not vary between experiments.

\*p < 0.01

## Discussion

In the laboratory setting, certain combinations of bile acids and amino acids are the most effective conditions for measuring *C. difficile* spore germination [84, 85, 129]. While cholic acid derivatives can stimulate *C. difficile* spore germination [85], CDCA-derivatives inhibit cholic acid-mediate germination

by *C. difficile* spores [95, 96]. Compared to humans, mice produce a low level of CDCA but produce other bile acids (AMA and BMA), in greater abundance. These muricholic acids may have an impact on how *C. difficile* spores germinate *in vivo*. Here, we found that AMA, BMA and OMA (a microbial product) inhibit taurocholic acid-mediated spore germination with BMA and OMA being the most potent germination-inhibiting muricholic acids (Table 1). These results are consistent with our previous work that has shown the 12-hydroxyl group to be an important determinant of whether a compound functions as a germinant or inhibitor of germination [85, 95, 96]. One difference observed between the germination of *C. difficile* UK1 spores and *C. difficile* M68 spores was the non-linear double-reciprocal plot for germination by *C. difficile* M68 spores. As seen in other strains, *C. difficile* M68 may bind taurocholic acid cooperatively [137]. With the recent identification of the molecular target of bile acids on the *C. difficile* spore, this hypothesis could be tested outright [98].

Total bile acid levels in the distal small intestine have been estimated to be between 1 mM to 2 mM in concentration [142]. This is in the range of the concentrations which inhibit *C. difficile* growth for the individual bile acids tested (Table 2); variations in pH may affect the toxicity of each bile acid [143]. Comparing these concentrations to the apparent  $K_i$  values determined for AMA and BMA, they are approximately 3x to 8x greater, respectively (Table 1). That is, in an antibiotic-treated mouse, the levels of AMA and BMA might prevent efficient *C. difficile* spore germination, possibly explaining why such greater

numbers of spores, compared to vegetative cells, are required to colonize a mouse [120]. It is also important to note that most mice used as a model for *C. difficile* infection would likely contain reduced levels of OMA because its formation requires the presence of mouse gut microbes [138, 139] which are likely 'collateral damage' during a routine course of broad-spectrum antibiotics.

Antibiotics can affect host functions. That is, treating mice with antibiotics could lead to alterations in the bile acid spectrum and increase or decrease the availability of activators or inhibitors of *C. difficile* spore germination. Treatment of mice with antibiotics has been shown to increase hepatic bile acid synthesis [144]. Specifically, the authors identified that small intestine, luminal concentrations of taurocholic acid, tauro- $\beta$ -muricholic acid and taurochenodeoxycholic acid were more abundant in antibiotic-treated C57/BL6 mice than in vehicle-only controls [144]; the authors did not measure the levels of AMA. Thus, upon antibiotic exposure, an increase in the abundance of germination-inhibiting bile acids could contribute to an environment which is more resistant to *C. difficile* spore germination.

Some *C. difficile* strains have been shown to stably colonize mice and enter a 'contagious' state, where disease is limited but spore shedding is maintained, while other strains are cleared by the host [113, 117]. The mechanisms by which some *C. difficile* strains are able to stably colonize a host while others do not, is unclear. While the answer is likely to be multifactorial, an increased resistance to bile acids could contribute to a strain's ability to persist

within a host. *C. difficile* M68 is a strain that can enter a supershedder state after the cessation of antibiotic treatment [113]. We find *C. difficile* M68 to be more resistant to bile acid toxicity than is *C. difficile* UK1 and this increased resistance may aid *C. difficile* M68 in maintaining active colonization.

Muricholic acids might provide a level of protection to mice from *C. difficile* infection that is not seen in other models of *C. difficile* disease. While our results suggest that particular bile acids may inhibit *C. difficile* spore germination or vegetative growth *in vitro*, it is unclear if AMA or BMA could substitute for each other in preventing *in vivo* spore germination. Clearly, BMA is a more potent inhibitor of *in vitro* spore germination than is AMA. But, given the vast repertoire of mouse lines and genetic approaches, testing the ability of *C. difficile* to colonize mice that have had introduced mutations into specific steps in the bile acid / muricholic acid synthesis pathway would allow the determination of which bile acids are relevant for stimulating or inhibiting *in vivo* spore germination and vegetative growth.

## CHAPTER III

### SPORE CORTEX HYDROLYSIS PRECEDES DPA RELEASE DURING *CLOSTRIDIUM DIFFICILE* SPORE GERMINATION\*

In Chapter III, we analyze how *C. difficile* spore germination differs from what has been observed during *B. subtilis* spore germination, a model organism. In particular, we determine when DPA is released from germinating spores and how this event is timed with the degradation of the spore cortex layer. In all other spore-forming bacteria studied to date, DPA release during spore germination occurs following germinant-germinant receptor interaction and the release of DPA triggers cortex hydrolysis. Based on the proposed location of the *C. difficile* germinant receptor, CspC, we proposed to that cortex hydrolysis occurred prior to DPA release and find that this is indeed true.

#### Introduction

*Clostridium difficile* (a Gram-positive, spore-forming, strict anaerobe) has become a significant threat to antibiotic-treated or immunocompromised hosts. Antibiotics are known to disrupt the colonic microbiota and this perturbation permits *C. difficile* colonization [145, 146]. Due to the strict anaerobic nature of *C. difficile* cells, spores are generally thought to be the infectious agent (only the spore can survive for extended periods of time in the aerobic environment

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outside of a host) [60, 147]. Because the spore form is non-infectious, spores must germinate to actively growing bacteria which initiate infection [98, 131]. Thus, germination by *C. difficile* spores represents one of earliest steps in the pathogenesis of this organism.

Endospore germination has been extensively studied in *Bacillus sp.* and, more recently, in clostridia [66, 97]. In the spore core, small acid soluble proteins help protect the chromosomal DNA and much of the water is replaced by pyridine-2,6-dicarboxylic acid (dipicolinic acid) as a 1:1 chelate with calcium (CaDPA) – accounting for approximately 10% of the dry weight of the spore [66]. Surrounding the spore core, is an inner spore membrane, a thin layer of cell wall peptidoglycan, a thick layer of specialized cortex peptidoglycan, an outer spore membrane and spore coat proteins. These features help protect the spore from environmental hardship and help the spore remain in a metabolically dormant state [66]. Even though spores are metabolically dormant, they interact with the environment and germinate when conditions become favorable for vegetative growth.

In *B. subtilis*, germinant receptor-mediated germination can be divided into two stages. Stage I is triggered when germinant receptors embedded within the inner spore membrane respond to the presence of small molecule germinants [66]. The most often described germinants for *B. subtilis* spores are L-alanine (or L-valine) or a mixture of L-asparagine, glucose, fructose and potassium ions (AGFK) [66]. The interaction of L-alanine / valine with the

GerAA-AB-AC germinant receptor or AGFK with the GerB / GerK germinant receptor leads to the release of CaDPA from the core, likely through the SpoVA channel, in exchange for water [66]. The release of CaDPA from the core completes stage I.

Stage II is activated by the release of CaDPA from the core during stage I, and stage II can be directly activated by an abundance of exogenous CaDPA (non-nutrient mediated spore germination) [66]. During stage II, cortex is degraded by the spore cortex lytic enzymes (SCLEs) CwlJ and SleB [66]. While the mechanism of activation of SleB is unknown, CwlJ activity is activated by DPA [66]. Thus, CaDPA release from the core stimulates cortex hydrolysis which leads to the swelling of the germ cell wall and core expansion. The expansion of the core results in further hydration of the core and complete CaDPA release [66]. Upon completing stage II, spores have lost most of their resistances and are no longer considered dormant. Then, in what has been described as 'ripening,' the germinated spore prepares for the outgrowth of a vegetative cell [148].

*C. difficile* spore germination is stimulated by a combination of cholic acid derivatives and glycine [85, 88] and inhibited by chenodeoxycholic acid derivatives [85, 95, 96, 98, 137]. While many of the ultrastructural features of the spore are conserved between *B. subtilis* and *C. difficile*, there are many differences [131, 149]. Significantly, *C. difficile* does not encode the classical *ger*-type germinant receptor [150]. Also, *C. difficile* encodes a single SCLE,



SleC [150, 151]. *C. difficile* SleC is synthesized in the mother cell during spore formation as a preprotein and *sleC* is required for colony formation by *C. difficile* spores [151, 152]. The pre-sequence is cleaved off, presumably during transport across the spore outer membrane. The proprotein remains inactive in the dormant spore until it is cleaved by a germination-specific protease, CspB [99]. In *C. difficile*, *cspB* is encoded as a fusion to *cspA* [99, 150]. Upon translation of the *cspBA* mRNA, CspBA undergoes interdomain cleavage to generate both CspB and CspA proteins [99]. A third protein, CspC, is encoded downstream of *cspBA* [99, 150]. In *C. perfringens*, all three Csp proteins have predicted catalytic activity (all three possess intact catalytic triads) [82, 83]. In *C. difficile*, only CspB is predicted to have catalytic activity because the residues important for catalysis are mutated in *cspA* and *cspC* [98, 99, 150]. Recently, we identified *C. difficile* CspC as the bile acid germinant receptor [98]. Certain SNPs in *C. difficile cspC* can abrogate spore germination while other SNPs alter germinant specificity [98]. We proposed a model where CspC activates CspB proteolytic activity and CspB cleaves pro-SleC to an active form. Activated SleC then begins to degrade the *C. difficile* spore cortex [98]. Because the *C. difficile* germinant receptor complex (CspA, CspB, CspC and SleC) is likely located in or near the spore cortex, while the *B. subtilis* germinant receptor complex is located in the spore's inner membrane, we hypothesized that there may be fundamental differences between the mechanisms of

germinant receptor-mediated *C. difficile* spore germination and *B. subtilis* spore germination.

Here we investigated how *C. difficile* spores germinate with respect to the proposed stages of germination, as described for *B. subtilis*. In contrast to what is observed for *B. subtilis* spore germination [and *C. perfringens* [81]], we found that cortex hydrolysis preceded DPA release during *C. difficile* spore germination. Significantly, mutations in either the *C. difficile* bile acid germinant receptor, CspC, or the cortex hydrolase, SleC, prevented both cortex hydrolysis and DPA release by germinating *C. difficile* spores. These results suggest that DPA release during *C. difficile* spore germination may be entirely dependent on core swelling or changes to cortex peptidoglycan and that the hypothesized glycine germinant receptor is likely not located in the spore inner membrane.

## **Materials and methods**

### **Bacteria and strains**

Wild-type *C. difficile* UK1 [96, 98, 153] and *C. difficile* M68 [113, 135, 153], *C. difficile* JSC10 (cspC::ermB) [98] and *C. difficile* CAA5 (sleC::ermB) were routinely grown in an anaerobic atmosphere (10% H<sub>2</sub>, 5% CO<sub>2</sub>, 85% N<sub>2</sub>) at 37°C in Brain Heart Infusion agar supplemented with 5 g / L yeast extract and 0.1% L-cysteine (BHIS). *B. subtilis* PS533 and *B. subtilis* FB113 (cwlJ::tet sleB::spc) [154] were a generous gift from Dr. Peter Setlow and were routinely grown on DSM medium. *E. coli* DH5α was grown on LB medium. Chloramphenicol (20 µg / ml), thiamphenicol (10 µg / ml), lincomycin (10 µg /

ml), kanamycin (50 µg / ml for *C. difficile*, 20 µg / ml for *E. coli*, 7 µg / ml for *B. subtilis*), spectinomycin (100 µg / ml) or tetracycline (5 µg / ml for *C. difficile*, 20 µg / ml for *B. subtilis*) were added where indicated.

### Molecular biology

To generate the TargeTron insertion into *C. difficile* UK1 *sleC* we took advantage of a previously described primer set [151]. The intron re-targeting fragment was generated using primers: *sleC* (128a) IBS (AAAAAAGCTTATAATTATCCTTACATTACTTCTTAGTGCGCCCAGATAGGGTG), *sleC* (128a) EBS1d (CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTCTTAGGTAACCTTCTTTGT), *sleC* (128a) EBS2 (TGAACGCAAGTTTCTAATTTTCGGTTTAATGTTCGATAGAGGAAAGTGTCT) and EBS universal (CGAAATTAGAACTTGCGTTCAGTAAAC) using SOE-PCR as describe in the TargeTron manual (Sigma-Aldrich, St. Louis, MO). The 350 bp fragment was cloned into pCR2.1-TOPO (Life Technologies, Carlsbad, CA) to yield pCA2 and the sequence of the insert verified. The 350 bp fragment was subcloned into the HindIII / BsrGI sites of the pJS107 TargeTron shuttle vector [153] to yield pJS113. The pJS113 plasmid was introduced into *B. subtilis* Bs49 using standard techniques. *B. subtilis* Bs49 pJS113 was introduced into *C. difficile* UK1 via conjugal transfer, as described previously [98]. Tetracycline-sensitive, thiamphenicol-resistant (Tn916 transposon-negative, plasmid-positive) strains were identified. These isolates were then

spread on BHIS medium supplemented with lincomycin to select for the TargeTron insertion into *sleC*. Lincomycin-resistant colonies were screened by PCR for the presence of the TargeTron insertion into *sleC*, as described previously [151]. Isolates with the insertion were frozen down as *C. difficile* CAA5 and have the expected phenotype of a *sleC* mutant (inability of spores to form colonies on BHIS agar supplemented with taurocholic acid) [151].

*C. difficile* CAA5 was complemented by expressing *sleC* *in trans* from the pJS116 shuttle vector (a pMTL84151 derivative) [98, 155]. The *C. difficile* UK1 *sleC* gene and promoter region were amplified using primers 5'sleC\_Gibson (TACGAATTCGAGCTCGGTACCCGGGGATCCGATTATTTTCCTTTCAAATT TTTGATTTATTTATGATTTATATC) and 3'sleC\_Gibson (AGTGCCAAGCTTGCATGTCTGCAGGCCTCGAGTTAAATTAAAGGATTTAA GAAGCTATTCTAGTTGTAG) and Phusion DNA polymerase (New England Biolabs, Beverly MA). The resulting fragment was introduced into pJS116 between the BamHI & XhoI restrictions sites using Gibson Assembly [156]. The resulting plasmid, pMF02, was introduced into *C. difficile* CAA5 as described above.

### Spore formation

*C. difficile* spores were generated as described previously [96, 98, 134, 153]. *B. subtilis* vegetative cells were spread on DSM agar medium for spore production [157]. After 2 days, growth was harvested by scraping the plates and suspending in water. This suspension (containing vegetative cells, cell debris

and spores) was then heated to 75°C for one hour to melt any agar that was scraped with spores. The suspension was centrifuged for 10 minutes at room temperature and 3,000 x g. The supernatant was removed and the pellet was resuspended in 10 mL of sterile water. To purify the spores from the vegetative cells and cell debris, the resuspended samples were layered on a gradient of 10 mL 20% HistoDenz (w / v), 10 mL 50% HistoDenz (w / v), and centrifuged for 1 hour at 4°C and 18,900 x g. The supernatant was then removed and the spore pellet was resuspended in 1 mL water. The purified spores were then washed 5 times in water by centrifuging for 1 minute at room temperature and 14,000 x g.

#### Monitoring the initiation of spore germination

*B. subtilis* spores and *C. difficile* spores were heat activated for 30 minutes at 80°C and 65°C, respectively, and then placed on ice. The initiation of spore germination was monitored aerobically at 600 nm (the initiation of *C. difficile* spore germination is unaffected by the presence of oxygen). To initiate *B. subtilis* spore germination, purified spores were suspended in 10 mM Tris, pH 8.4 and 100 mM L-valine. *C. difficile* spore germination was initiated by suspending spores in 10 mM Tris, pH 7.5, 150 mM NaCl, 100 mM glycine and 10 mM taurocholic acid.

Spores were heat shocked at either 80°C for *B. subtilis* or 65°C for *C. difficile* for 30 minutes. 5 uL of spores were diluted into 995 uL of buffer with or without germinant, mixed and the change in optical density at 600 nm was measured.

### Monitoring CaDPA release

CaDPA release was monitored in real time using terbium fluorescence [158]. An opaque, 96-well plate was prepared with the 125  $\mu$ L of the germination solutions (see above) supplemented with 800  $\mu$ M TbCl<sub>3</sub>. Heat-activated spores were then sedimented for 1 minute at 14,000 x g and resuspended in an equal volume of water to remove any CaDPA that may have released due to autogerminating spores. A 5  $\mu$ L sample of a 60 OD<sub>600</sub> spore suspension was added to each well and CaDPA release monitored using a Molecular Devices Spectramax M3 fluorescence plate reader (Molecular Devices, Sunnydale, CA) (excitation: 270 nm, emission: 545 nm, cutoff: 420 nm – appropriate wavelengths for the DPA-Tb<sup>3+</sup> complex). For experiments involving mutations in the germination pathway (i.e., *B. subtilis* *cwlJ/sleB* or *C. difficile* *cspC* or *C. difficile* *sleC*), the amount of CaDPA released was compared to that of the WT strain.

### Assaying cortex fragment release by germinating spores

Cortex fragments were detected using an assay based on the presence of reducing sugars in the germination medium, as described previously [159, 160]. Briefly, *B. subtilis* spores or *C. difficile* spores were heat activated, as described above, and stored on ice until use. An 11 ml germination solution (see above) was prepared. Before beginning the assay, a 1.0 mL sample was drawn to serve as a blank for cortex fragment detection and a separate 100  $\mu$ L sample was taken as a blank for measuring DPA release. A target spore density

of  $OD_{600} \approx 3.0$  yielded the best results for detecting cortex fragments. A zero time point sample was taken immediately after the addition of spores and centrifuged for 1 minute at  $14000 \times g$ . 1.0 mL of this sample was transferred to a fresh tube for cortex fragment analysis (see below) and 100  $\mu$ L was taken to monitor the amount of CaDPA released. This procedure was repeated at selected time points until the experiment was completed. After all time points were collected, samples were frozen at  $-80^{\circ}\text{C}$  and lyophilized.

Lyophilized samples were resuspended in 120  $\mu$ L of 3N HCl supplemented with 1% phenol and 0.5%  $\beta$ -mercaptaethanol and then transferred to 2 mL screw-cap tubes. Samples were then placed in a  $95^{\circ}\text{C}$  recirculating water bath for 4 hours. After incubation, the samples were placed on ice until cool and neutralized with 120  $\mu$ L of 3M NaOH. To those samples, 80  $\mu$ L of a saturated sodium bicarbonate solution and 80  $\mu$ L of a 5% acetic anhydride solution were added and the samples were mixed. Samples were incubated at room temperature for 10 minutes then transferred back to the  $95^{\circ}\text{C}$  water bath for 3 minutes. Samples were removed from the water bath, cooled on ice, 400  $\mu$ L of 6.54%  $\text{K}_2\text{B}_4\text{O}_7 \cdot 4\text{H}_2\text{O}$  was added to each tube and then mixed. The resulting solution was then heated for 7 minutes in the  $95^{\circ}\text{C}$  water bath and then placed on ice for 5 minutes during which the color reagent was made. This color reagent was made by dissolving 0.320 g p-dimethylaminobenzaldehyde in 1.9 mL glacial acetic acid. After the p-dimethylaminobenzaldehyde was completely dissolved, 100  $\mu$ L of 10N HCl was added, the solution was mixed and

then 5 mL of glacial acetic acid was added. 100  $\mu$ L of each cooled cortex sample was transferred to a new 1.5 mL microcentrifuge tube and 700  $\mu$ L of the color solution was added. Samples were incubated in a 37°C water bath for 20 minutes. Following incubation, 200  $\mu$ L of each sample was transferred to a clear 96-well plate and quantified at 585 nm using a Molecular Devices Spectramax M3 fluorescence plate reader (Molecular Devices, Sunnydale, CA). As a positive control for reducing sugar detection, in each experiment a standard curve was generated using 0, 12.5, 25, 50, 100, 250, 500 and 5000 nmol N-acetylglucosamine. For experiments involving mutations in the germination pathway (i.e., *B. subtilis* *cwlJ/sleB* or *C. difficile* *cspC* or *C. difficile* *sleC*), the amount of cortex released was compared to that of the WT strain.

#### Statistical analysis

Data points represent the mean from three independent experiments and error bars represent the standard deviation from the mean. Statistical analysis between time points, where indicated, was performed using a two-tailed Student's T-test.

### **Results**

#### Comparing the initiation of *C. difficile* and *B. subtilis* spore germination

*B. subtilis* spore germination can be triggered via several pathways (e.g., nutrient-mediated activation of the *ger*-type germinant receptors or direct activation of cortex hydrolysis by CaDPA). To begin to understand the events that occur during *C. difficile* spore germination, we compared *C. difficile* spore



germination to that of *B. subtilis* spore germination via activation of their respective germinant receptors. During spore germination, spores transition from a phase-bright state (dormant) to a phase-dark state (loss of dormancy). This transition can be monitored spectrophotometrically by measuring the optical density at 600 nm of pure spore suspensions incubated under different conditions. When spores respond to germinant, they release their large depot of CaDPA from the core. This action results in a large and rapid decrease in the OD<sub>600</sub> of the spore suspension [161]. *C. difficile* UK1 spores were suspended in buffer supplemented with either taurocholic acid and glycine or taurocholic acid alone and germination was monitored at 600 nm. As described previously, spores rapidly germinated upon exposure to both taurocholic acid and glycine but not when exposed to taurocholic acid alone (Figure 8A, data not shown) [85]. While the use of absorbance to monitor germination is convenient, it is not a quantitative measure of CaDPA release and can include cortex hydrolysis at later time points [161]. Thus, to provide a quantitative measure of DPA release, we monitored CaDPA release in real-time using an assay based on terbium fluorescence [158, 162]. *C. difficile* spores released CaDPA in the presence of taurocholic acid and glycine but not in response to taurocholic acid alone (Figure 8A, data not shown) and completed DPA release in approximately 30 minutes (no further increase in DPA occurred after 30 minutes).

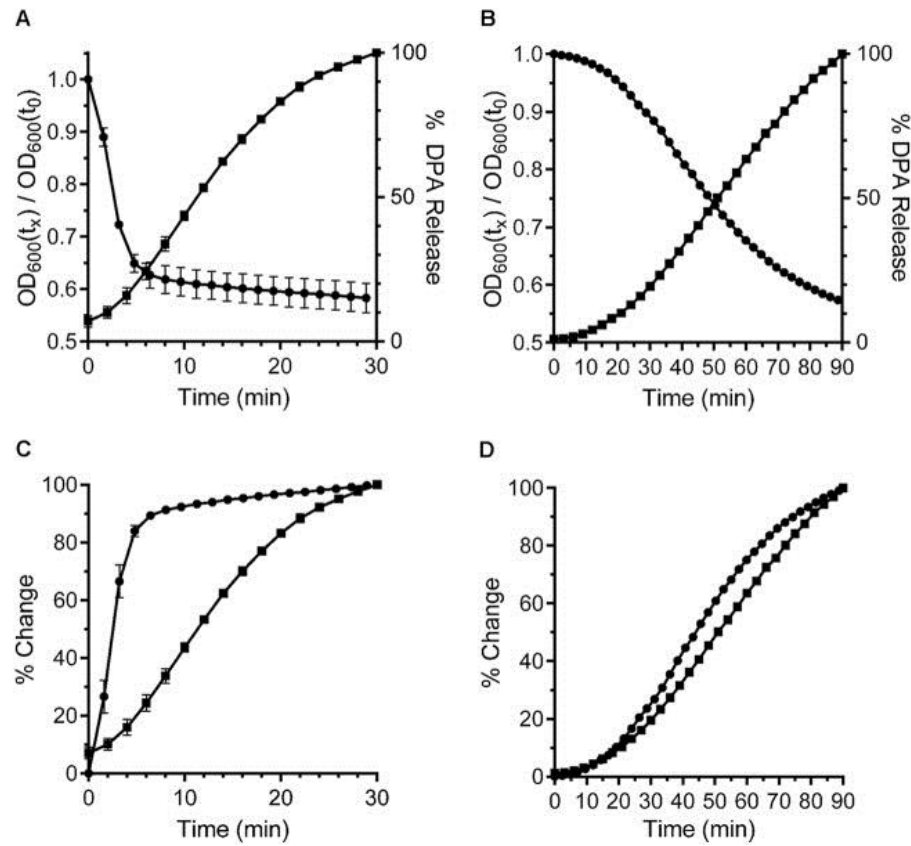


Figure 8. Comparison of the initiation of *C. difficile* and *B. subtilis* spore germination.

(A) Purified *C. difficile* UK1 spores were suspended in buffer supplemented with taurocholic acid and glycine. Germination was monitored by plotting the ratio of the  $OD_{600}$  at a given time to the  $OD_{600}$  at time zero (●) and DPA release from germinating *C. difficile* spores was monitored using  $Tb^{3+}$  fluorescence and normalized to the maximum amount of DPA released in the indicated time frame (■). (B) Purified *B. subtilis* PS533 spores were suspended in buffer supplemented with L-valine and germination was monitored as described above. The data from the  $OD_{600}$  in panels A and B were converted to the percent change so that the curves could be directly compared. The converted  $OD_{600}$  data were plotted with the DPA release data in panels C and D, respectively. The data represent the averages from three independent experiments, and error bars represent the standard deviations.

To compare *C. difficile* spore germination to that of *B. subtilis*, we incubated purified *B. subtilis* spores in buffer supplemented with L-valine. As described previously, the absorbance of the spore suspension decreased when incubated in the presence of L-valine and not in the absence of L-valine (Figure 8B, data not shown) [163]. Similarly, Tb<sup>3+</sup> fluorescence increased when *B. subtilis* spores were suspended in buffered L-valine (indicating CaDPA release) but not in buffer alone (Figure 8B, data not shown).

So that we could directly compare the absorbance assay to the terbium fluorescence assay, we plotted the percent change from the absorbance assay and the percent maximum Tb<sup>3+</sup> fluorescence on the same graph. When analyzed in this manner, changes in the absorbance of germinating *C. difficile* spores occurred much earlier than changes observed in Tb<sup>3+</sup> fluorescence (CaDPA release) (Figure 8C). During *B. subtilis* spore germination, the CaDPA release curve closely followed that of the absorbance curve, confirming a previous study which demonstrated that much of the absorbance change is due to the released CaDPA. (Figure 8D) [161]. Because CaDPA release is one of the first measurable events during germinant receptor-activation of *B. subtilis* spore germination, these results suggest that there may be events occurring during *C. difficile* spore germination before CaDPA is released.

CaDPA release precedes cortex hydrolysis release during *B. subtilis* spore germination

When CaDPA is released from the spore core, it transits through the spore cortex and activates the spore cortex hydrolase, CwlJ. CwlJ activity (and SleB activity) leads to the release of cortex fragments into the surrounding germination medium. *B. subtilis* spores suspended in germination buffer supplemented with L-valine released most of their CaDPA within 2.5 hours (Figure 9A). Spores suspended in buffer alone did not release CaDPA (data not shown). When we monitored for the presence of cortex fragments (as measured by the abundance of reducing sugars in the germination solution), we observed that these cortex fragments appeared after CaDPA is released (Figure 9A) and their presence was dependent on L-valine (data not shown). These results confirm the previous observations that CaDPA release precedes cortex hydrolysis in *B. subtilis* and, importantly, that we can detect cortex fragments during spore germination [161].

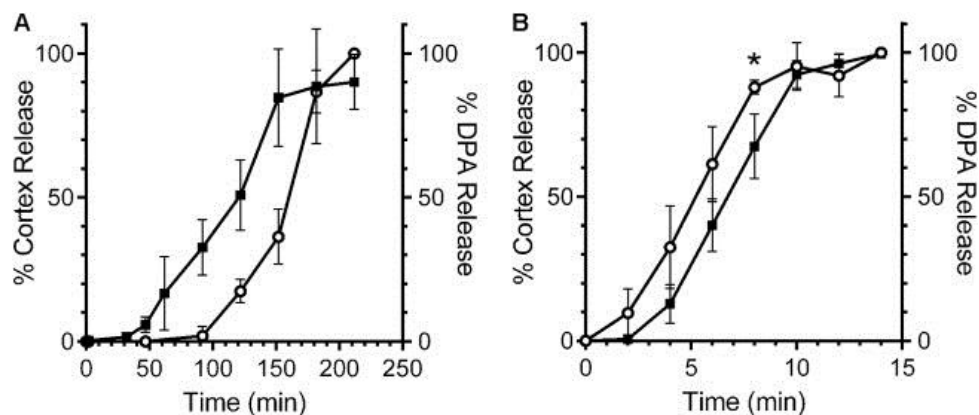


Figure 9. Comparing the release of cortex fragments and CaDPA from germinating *B. subtilis* and *C. difficile* spores. (A) Purified *B. subtilis* PS533 spores were suspended in buffer with l-valine. (B) Purified *C. difficile* UK1 spores were suspended in buffer with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solutions were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations. \*,  $P < 0.04$ .

#### Cortex hydrolysis precedes CaDPA release during *C. difficile* spore germination

Based on our observations that the optical density of germinating *C. difficile* spores decreased before the appearance of CaDPA in solution (Figure 8C) and that the newly identified bile acid germinant receptor is likely cortex-localized [98], we hypothesized that cortex hydrolysis may precede CaDPA release during *C. difficile* spore germination. *C. difficile* UK1 spores were suspended in germination buffer supplemented with taurocholic acid only or both taurocholic acid and glycine and assayed for the presence of both cortex fragments and DPA in the germination solution. Interestingly, we detected cortex fragment release from the germinating spores within 2 minutes of

germination (the earliest time point we can measure). At this time point, CaDPA is either not released or below the limit of detection (Figure 9B). At four minutes, the rate of cortex fragment release increased. During this time CaDPA begins to be released and followed closely the curve for the cortex fragments, which remained steady until 8 minutes after the initiation of germination (Figure 9B). At 8 minutes, there was a statistically significant difference between the cortex fragment curve and the CaDPA curve ( $p\text{-val} < 0.04$ ). Taken together, these results suggest that *C. difficile* spore cortex hydrolysis precedes CaDPA release during germination.

To control for the observed differences in cortex hydrolysis and CaDPA release, we analyzed cortex hydrolysis in *B. subtilis* FB113, a strain with engineered mutations in both cortex hydrolases (*sleB/cwlJ*). When *B. subtilis* FB113 spores were suspended in buffer supplemented with L-valine, spores released CaDPA (Figure 10A). However, inactivating both *sleB* and *cwlJ* cortex hydrolases resulted in the inability of these *B. subtilis* spores to hydrolyze cortex in response to L-valine (Figure 10A).

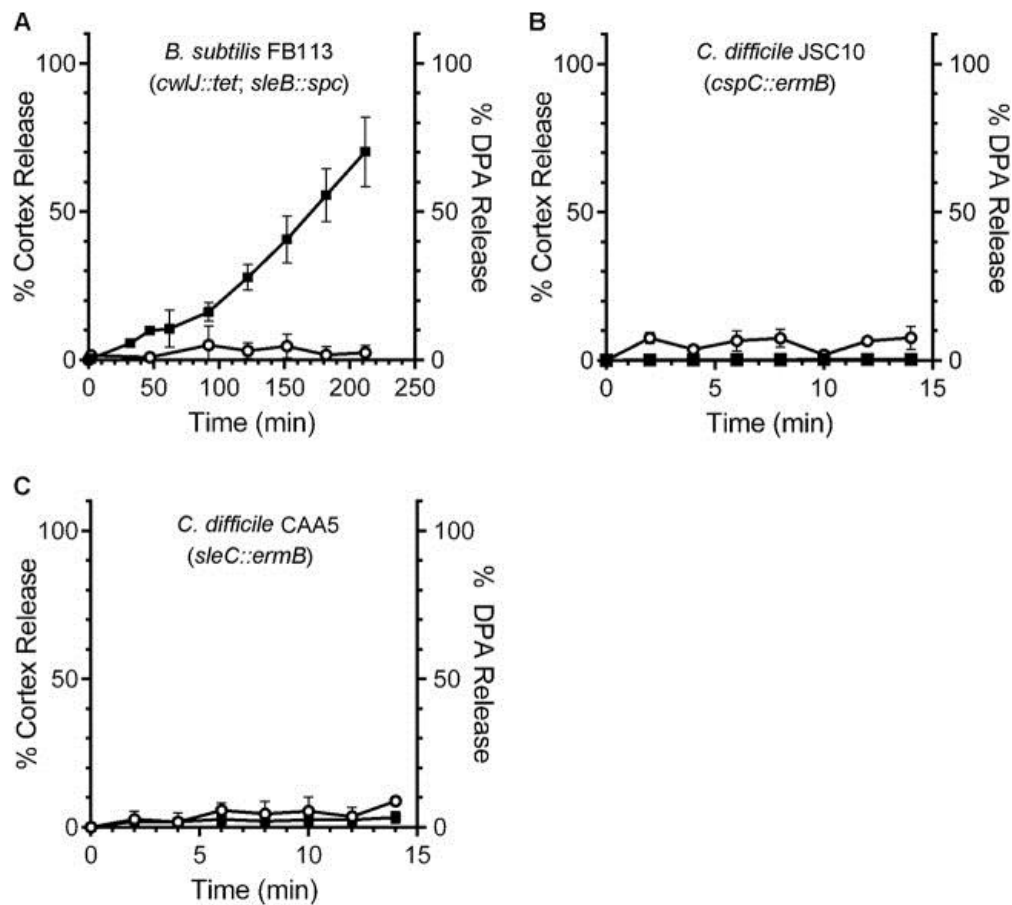


Figure 10. Genetic analysis of cortex hydrolysis and CaDPA release from germinating *B. subtilis* and *C. difficile* spores. (A) Purified *B. subtilis* FB113 (*cwlJ::tet sleB::spc*) spores were suspended in buffer supplemented with l-valine. (B and C) *C. difficile* JSC10 (*cspC::ermB*) spores (B) and *C. difficile* CAA5 (*sleC::ermB*) spores (C) were suspended in buffer supplemented with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solution were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations.

Conversely, when *C. difficile cspC* spores are suspended in buffer supplemented with taurocholic acid and glycine, neither cortex fragments nor CaDPA are released (Figure 10B); CaDPA release was restored by expressing

*cspBAC in trans* [98]. Further, inactivating the lone *C. difficile* SCLE, *sleC*, also prevented cortex hydrolysis and CaDPA release (Figure 10C); germination (both by OD<sub>600</sub> and CaDPA release) was restored by expressing *in trans* a copy of *C. difficile sleC* (Figure 11). Because *C. difficile cspC* still expresses the SleC cortex hydrolase, but CaDPA and cortex fragments are not released, these results suggest that cortex hydrolysis and CaDPA release during *C. difficile* spore germination are coupled.

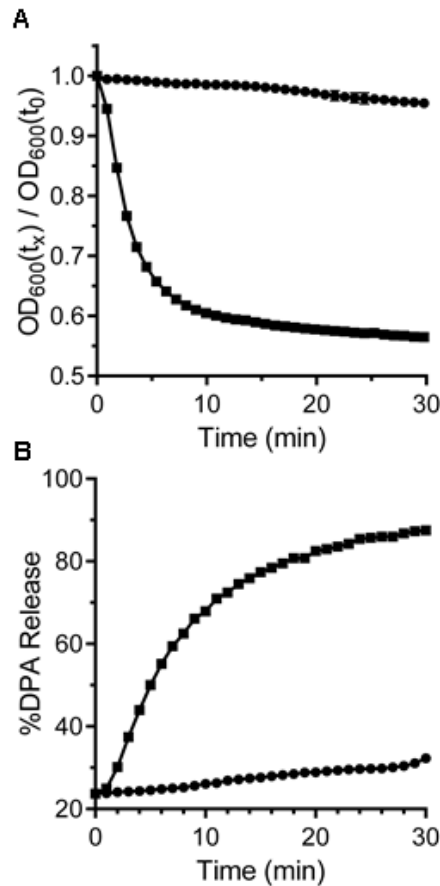


Figure 11. Complementing the *C. difficile sleC* mutation *in trans*.



### Analyzing spore germination in another *C. difficile* strain

It was previously reported that there may be heterogeneity among *C. difficile* isolates in terms of their germination responses [164, 165]. Therefore, we analyzed how cortex hydrolysis and CaDPA release occurs in another *C. difficile* ribotype. As described above for *C. difficile* UK1, when *C. difficile* M68 spores are suspended in buffer containing taurocholic acid and glycine [but not taurocholic acid only (data not shown)], cortex fragments appeared in the germination solution before CaDPA is detected (Figure 12). During *C. difficile* M68 spore germination, at the earliest time point of 2 minutes, there was a difference between cortex fragments and CaDPA ( $p\text{-val} < 0.001$ ) (Figure 12). These results support the idea that cortex hydrolysis preceding CaDPA release is a general phenomenon during *C. difficile* spore germination and not specific to one isolate. Our results clearly show that the initiation of germinant receptor-mediated *C. difficile* spore germination occurs through a novel pathway.

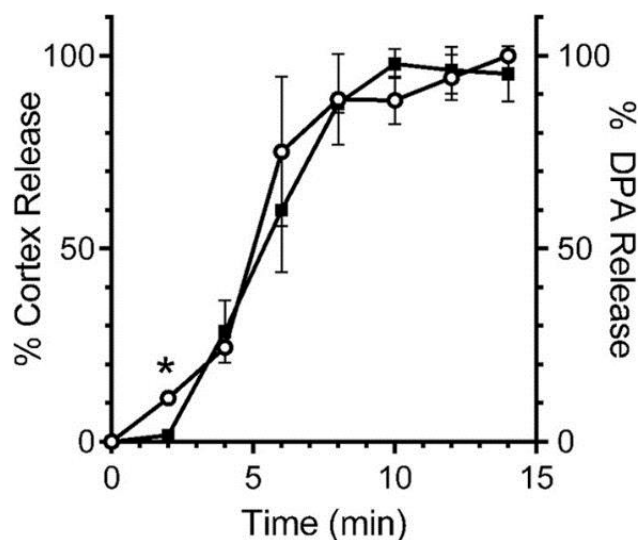


Figure 12. Cortex hydrolysis precedes CaDPA release during *C. difficile* M68 spore germination. Purified *C. difficile* M68 spores were suspended in buffer with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solution were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations. \*,  $P < 0.001$ .

## Discussion

Germination by *C. difficile* spores seems to occur differently than for other spore-forming bacteria. Upon sequencing and analysis of the *C. difficile* genome, it was apparent that *C. difficile* did not encode orthologues of the *ger*-type germinant receptors found in other spore-forming bacteria. This suggested that *C. difficile* spores may germinate in response to unique germinants or use novel mechanisms to initiate spore germination or both [150]. It has been known for approximately 30 years that certain bile acids stimulate *C. difficile* spore germination [Wilson, 1982 #24; [166]. Though much work has

focused on the signals that can stimulate or inhibit *C. difficile* spore germination, the proteins that responded to these signals had remained elusive. In a genetic screen to select for *C. difficile* mutants whose spores do not respond to taurocholic acid as a germinant, we identified the CspC as the bile acid germinant receptor [98]. Due to the differences between the predicted locations of the *C. difficile* germinant receptor complex (CspC, CspB and SleC) and the locations of the *B. subtilis* spore germinants receptors (GerAA-AB-AC) we hypothesized that *C. difficile* spore germination may occur differently than that observed in the model organism.

Here we observe that *C. difficile* spore germination is not initiated in the same manner as observed for *B. subtilis*. We find that cortex hydrolysis precedes CaDPA release during germination by *C. difficile* spores and that this seems to be a general phenomenon among *C. difficile* isolates; the *C. difficile* M68 strain, a different ribotype, also released cortex fragments before CaDPA. Unlike what is observed for *B. subtilis* spore germination, we could not genetically separate cortex hydrolysis from CaDPA release by inactivating either the bile acid germinant receptor or the SCLE. Both *C. difficile* *cspC* and *C. difficile* *sleC* are required to hydrolyze cortex and without cortex hydrolysis the release of CaDPA from the core in is not observed (Figure 10B and Figure 10C, respectively).

Interestingly, *C. perfringens* encodes orthologues of both the classical *ger*-type germinant receptor, the Csp proteases and SleC. However, *C.*

*perfringens* does not germinate in response to bile acids [78, 167]. Though there seems to be conservation in the Csp proteases, *C. perfringens* CspA, CspB and CspC are catalytically active proteases which could activate SleC to stimulate cortex hydrolysis [82, 83, 159]. Mutations in *C. perfringens* *sleC* result in strains that still release CaDPA but do not hydrolyze cortex [81]. Thus, our observation that cortex hydrolysis precedes CaDPA release during *C. difficile* spore germination is not a general phenomenon among all Clostridia. But, rather, that this may be a novel mechanism for stimulating germination in spore-forming bacteria that do not encode the classical *ger*-type germinant receptor.

If mutations in the bile acid germinant receptor prevent both cortex hydrolysis and CaDPA release from the core, how is CaDPA release mediated during *C. difficile* spore germination? In our working model (Figure 13), taurocholic acid interacts with CspC which transmits the bile acid signal to CspB. Activated CspB, in turn, cleaves proSleC to an active hydrolase which begins to hydrolyze cortex, releasing cortex fragments into the surrounding milieu. Cortex hydrolysis allows the germ cell wall to expand and, with it, the inner spore membrane. In our model, either an unidentified protein responds to the cell wall expansion and triggers CaDPA release, or the expansion of the inner spore membrane alone triggers CaDPA release. This would suggest a mechanosensitive channel is responsible for release CaDPA during *C. difficile* spore germination. *C. difficile* encodes orthologues of several mechanosensitive proteins (e.g. *mscL* and *mcsS*) and most of these are likely to be involved in

maintaining osmotic homeostasis during vegetative growth and probably have no role in spore germination [150, 168-170].

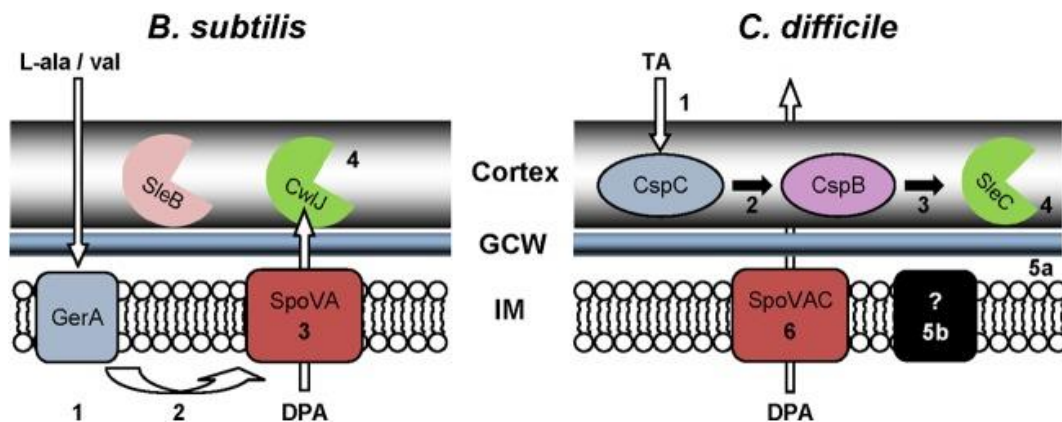


Figure 13. Models for spore germination.

(A) During the initiation of *B. subtilis* spore germination, L-alanine (or L-valine) interacts with the GerA germinant receptor complex (location 1). The SpoVA channel (which includes the SpoVAD DPA-binding protein) is then activated (location 2), and it releases CaDPA from the spore core (3). Released CaDPA activates the CwlJ cortex hydrolase (location 4) triggering cortex hydrolysis. (B) The initiation of *C. difficile* spore germination is triggered when the bile acid germinant receptor, CspC, interacts with taurocholic acid (location 1). Activated CspC then activates the germination-specific protease, CspB (2), which processes pro-SleC to an active form (location 3), and cortex hydrolysis begins (location 4). Then, due to either core swelling (location 5a) or through the action of an unknown protein (location 5b), SpoVAC releases CaDPA (location 6).

In *B. subtilis*, germination is triggered through the interaction of germinants with the germinant receptors imbedded in the inner spore membrane (Figure 13). The interaction of these germinants with their cognate receptors triggers CaDPA release, likely through the SpoVA channel (Figure 13) [72].

Then, as described above, CaDPA activates cortex hydrolysis (Figure 13). The *B. subtilis* SpoVA complex is composed of 7 different proteins: SpoVAA, SpoVAB, SpoVAC, SpoVAD, SpoVAEa, SpoVAEb and SpoVAF. Most of these proteins are important for both CaDPA import into the developing spore during sporulation and CaDPA release during germination [e.g. SpoVAD binds DPA [54]]. *C. difficile* does not encode orthologues of many of these proteins. However, *C. difficile* does encode *spoVAC*, *spoVAD* and *spoVAE*. Recently, Velásquez and colleagues reported a function for SpoVAC [53]. In their manuscript, the authors determined that SpoVAC is a mechanosensitive channel [53]. How this protein functions during *B. subtilis* spore germination is unclear. For *C. difficile* spore germination, we propose that SpoVAC responds to the change in osmolarity that occurs upon cortex hydrolysis and then either, itself, provides a channel for CaDPA release or is part of a larger channel which is mechanically gated.

While the *C. difficile* bile acid germinant receptor is known, with what glycine interacts is not. We speculated that the unidentified glycine germinant receptor may be localized to the inner membrane of the spore core thus providing a similar function as the *B. subtilis* germinant receptors [98]. However, because a mutation in the bile acid germinant receptor prevented both cortex hydrolysis and CaDPA release, we propose that the hypothesized glycine receptor to be either: i) part of the known *C. difficile* germinant receptor complex

(CspB, CspA, CspC and SleC) or ii) located in the inner spore membrane but whose activity is dependent on CspC activity.

## CHAPTER IV

### DIPICOLINIC ACID RELEASE BY GERMINATING *CLOSTRIDIUM DIFFICILE*

#### SPORES OCCURS THROUGH A MECHANOSENSING MECHANISM\*

In Chapter IV, we build upon our findings in Chapter III and analyze the mechanism of DPA release during *C. difficile* germination. Prior studies in *B. subtilis* had demonstrated that SpoVAC was a mechanosensing protein and essential for DPA packaging and release. Because *C. difficile* encodes a *spoVAC* orthologue, we hypothesized that DPA release could occur in response to the changes in osmolarity that are a result of the degradation of the spore cortex layer. To test this hypothesis, we germinated spores under high solute conditions and analyzed these effects on the release of DPA and cortex fragments during *C. difficile* spore germination.

### Introduction

*Clostridium difficile* is a Gram-positive, spore forming, strict anaerobe that most-commonly infects immunocompromised or antibiotic treated hosts. Most antibiotics have broad spectrum activity which disrupts the normal microbiota that provides colonization resistance to *C. difficile* infection [171]. This disruption enables *C. difficile* to colonize and cause disease [124]. In a host, *C. difficile* secretes two toxins (TcdA and TcdB) that damage the colonic epithelium and

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elicit the primary symptoms of disease [172]. Though disease is caused by vegetative cells, it is the spore form that is responsible for transitioning the aerobic environment between hosts [60].

Spores are metabolically dormant forms of bacteria that are resistant to many harsh conditions (e.g., heat, desiccation and antibiotics) [97, 101]. The spore structure is conserved across most spore-forming, Gram-positive bacteria and is important for maintaining the spore's resistance properties. Contained within the spore core is the genomic DNA bound by small acid soluble proteins (SASPs) and a large quantity of Ca-dipicolinic acid (DPA) [66, 169]. The SASPs protect the DNA from UV damage and DPA packaging helps protect the core from heat by excluding water [20, 48, 173]. Surrounding the core is an inner spore membrane, where many of the Ger-type germinant receptors found in Bacilli, and most clostridia, are located [97]. Surrounding the inner membrane is a thin layer of germ cell wall peptidoglycan and a thick layer of specialized cortex peptidoglycan. In cortex peptidoglycan, many of the N-acetylmuramic acid residues have been converted to muramic- $\delta$ -lactam residues and are the targets for cortex degrading enzymes [66]. An outer membrane surrounds the cortex and functions as a scaffold with which to build the coat layer. In some spore-forming bacteria, including *C. difficile*, an additional exosporium layer surrounds the spore coat [97].

Spores remain metabolically dormant until specific signals, germinants, are detected by receptors (germinant receptors) in the spore. Germinant

recognition by germinant receptors leads to the irreversible initiation of the germination process. In *B. subtilis*, a model organism for studying sporulation and germination, the L-alanine germinant is recognized at the inner spore membrane by the GerA germinant receptor (which is composed of the GerAA-AB-AC proteins) while L-asparagine, D-glucose, D-fructose, and K<sup>+</sup> ions (AGFK) are recognized by GerB and GerK (which are composed of GerBA-BB-BC and GerKA-KB-KC, respectively) [66]. The activation of these germinant receptors triggers the release of cations and DPA from the core. The mechanism of DPA release is unclear, however the proteins encoded by the *spoVA* operon (SpoVAA-AB-AC-AD-AEa-AEb-AF) play a role [53-55, 72, 174, 175]. In *B. subtilis*, the release of DPA activates the spore cortex lytic enzyme (SCLE), CwlJ, and the actions of CwlJ and SleB lead to cortex degradation [66]. This mechanism of spore germination is similar across most spore-forming bacteria studied to date.

*C. difficile* spore germination is triggered by a combination of certain bile acids and amino acids [85, 95, 96, 101, 130]. In contrast to the mechanisms of germination observed in Bacilli and most Clostridia, *C. difficile* does not encode the Ger-type germinant receptors [150]. Instead, *C. difficile* uses the germination-specific, pseudoprotease, CspC, as the bile acid germinant receptor [98]. In *Clostridium perfringens*, CspA, CspB and CspC are active proteases with the potential to cleave the SCLE, pro-SleC, to its active form [81-83, 101, 176-178]. Interestingly, *C. difficile* CspA and CspC are pseudoproteases, their

catalytic triads are not complete. Due to the apparent lack of catalytic activity, we proposed a working model where activated CspC signals CspB to cleave pro-SleC to an active form. SleC activation initiates cortex degradation. Recently, another protein, GerS, was identified to play an important role during *C. difficile* spore germination [100]. Spores lacking GerS fail to degrade cortex but still process SleC into its active form [100]. In contrast to the mechanisms of germination observed in *B. subtilis*, during *C. difficile* spore germination the DPA contained within the core is released after cortex degradation begins [179]. Whereas *B. subtilis* releases DPA through a pore presumably formed by the proteins encoded by the *spoVA* operon, *C. difficile* does not encode the entire operon [150, 180]. Instead, *C. difficile* encodes three homologues: *spoVAC*, *spoVAD* and *spoVAE* [180]. In *B. subtilis*, the *spoVA* proteins are required for the completion of sporulation, likely due to defects in DPA packaging [52]; SpoVAD is thought to act as a DPA binding protein [54], helping to package DPA in the core during spore formation. Moreover, a recent study identified a mobile genetic element that encodes *spoVAC*, *spoVAD* and *spoVAE* and the resulting overexpression of these proteins lead to accumulation of up to 50% more DPA in the spores and an increased heat resistance [173].

*B. subtilis* SpoVAC was shown to have mechanosensing properties [53]. If SpoVAC functions as a mechanosensing protein in *C. difficile*, it could respond to the changes in osmolarity observed at the inner spore membrane due to the removal of constraints placed upon the dormant, dehydrated core by the cortex

layer. Cortex degradation may allow pores to open in response to the lower osmotic pressure of the environment, relative to the DPA rich core. We hypothesized that a spore germinating in an environment with an osmolyte concentration equal to or higher than that of the core would affect DPA release. Here, we investigated the role high osmolyte concentrations have on cortex degradation and DPA release during *C. difficile* spore germination. We find that high osmolyte concentrations can block DPA release from the core while permitting cortex degradation. Our data suggest that DPA release during germination by *C. difficile* spores is due to changes in osmolarity that occur during cortex degradation.

## **Materials and methods**

### Bacteria and strains

Wild-type *C. difficile* R20291 and *C. difficile* M68, were routinely grown at 37°C in an anaerobic atmosphere (10% H<sub>2</sub>, 5% CO<sub>2</sub>, 85% N<sub>2</sub>) on brain heart infusion agar supplemented with 5 g / L yeast extract and 0.1% L-cysteine (BHIS). *E. coli* DH5 $\alpha$ , *E. coli* HB101 pRK24 and *B. subtilis* BS49 were grown on Luria-Bertani (LB) medium supplemented with antibiotics as needed. Chloramphenicol (20  $\mu$ g / ml), thiamphenicol (10  $\mu$ g/ml), kanamycin (50  $\mu$ g / ml for *C. difficile*, 20  $\mu$ g / ml for *E. coli*), or tetracycline (5  $\mu$ g / ml for *C. difficile* and 20  $\mu$ g / ml for *B. subtilis*) were added where indicated.

## Molecular biology

Using the *codA*-dependent allelic exchange strategy [181], we engineered a deletion of *spoVAC* in *C. difficile* R20291. To do so, we inserted the Tn916 *oriT* inserted into pMTL-SC7215, using primers pMTL\_SC\_7215\_tn916\_L (ctagagtcgacgtcacgcgtccatggagatctcgagtaacatcttctattttcccaaactcttac) and pMTL\_SC\_7215\_tn916\_R (ggccagtgccaaagcttgcattgtctgcaggcctcgagctaaaggaatgtagataaattattaggaatctgc), to make pMF12, as described previously [98]. *C. difficile* R20291 DNA was used as a template to amplify 1kb upstream and downstream of the *spoVAC* deletion using primers spoVAC\_ndel\_L (agctatgaccgcggccgctgtatccatgatgagttcaaaatggagatgaagaggcaaaaga) spoVAC\_LHF\_Rev\_II (ctaaaacatcttaaaaatataataaataatgtctacatattttataattttatcca) spoVAC\_xhoI\_L (atggataaaaaattataaaaaatgttagacattattattatattttaagatgttttagatgat) spoVAC\_xhoI\_R (tgccaagcttgcattgtctgcaggcctcgagggtctttaagggttaaacatctctataccac). The resulting 1 kb fragments were stitched together using SOE PCR and subcloned into pMF12 digested with *NdeI* / *XhoI*, yielding pMF11. The pMF11 plasmid was introduced into *B. subtilis* BS49 using standard techniques. Subsequently, the pMF11 plasmid was introduced into *C. difficile* R20291 via conjugal transfer from *B. subtilis* BS49 pMF11, as described previously [98]. Tetracycline-sensitive, thiamphenicol-resistant (transposon-negative, plasmid-positive) strains were

identified. These isolates were then spread on BHIS supplemented with kanamycin and thiamphenicol to enrich for and identify faster growing single-crossover integrant clones. The larger colonies were then selected and plated on CDMM supplemented with 50 µg / ml 5-fluorocytosine (FC). The colonies formed after 48 hours were tested for thiamphenicol sensitivity by culturing and by PCR with primers 5'catP3 (atggtatttgaaaaaattgataaaaatag) and 3' catP2 (ttaactatttatcaattcctgcaattcg) to confirm loss of the plasmid. To confirm the deletion in *spoVAC*, the colonies were screened by PCR amplification of the *spoVAC* surrounding region using *spoVAC\_ndeI\_L* and *spoVAC\_xhoI\_R*. In order to generate the *spoVAC*-complementing plasmid, *C. difficile* *spoVAC* was amplified using Phusion polymerase with the 5'spoVAC\_Gibson (catgattacgaattcgagctcggtacccggggatcctaatacttatgatatgtagaataacaaaatataataaatatattact) and 3'spoVAC\_Gibson (ccagtgccaaagcttgcattgtctgcaggcctcgagatctagtggtggtggtggtgtagagtatttgctatctgttgaatcgtat) oligonucleotides. The resulting fragment was cloned by Gibson Assembly [156] between the *Bam*HI and *Xho*I restriction sites of the *B. subtilis* – *C. difficile* shuttle vector, pJS116, to generate pMF15. The nucleotide sequences were confirmed before use.

### Spore formation

*C. difficile* strains were plated on reduced BHIS plates and allowed to grow for 4 days, as described previously [85, 96, 179]. On day 4, growth was

harvested from two plates by scraping the plates with a disposable inoculating loop and suspended into 1.5 mL microcentrifuge tubes containing 1 mL sterile water. Tubes were stored at 4 °C overnight. The next day, the contents of each tube were resuspended through pipetting and immediately centrifuged for 1 minute at 14,000 x g. The supernatant was removed and the pellet was resuspended in water and centrifuged for 1 minute at 14,000 x g and was repeated for a total of 5 times. After resuspending the pellet again in 1 mL of water, the contents of 2 tubes (2 mL total suspension) were carefully layered over 8 mL of 60% sucrose (w / v). This was centrifuged for 20 minutes at 4,000 x g. Subsequently, the supernatant was removed and the remaining pellet was suspended in 1 mL of sterile water. As above, spores were washed 5 times in sterile water. After the final wash, the supernatant was removed and the purified spore preparations were combined in 1 mL of sterile water. After purification, the resulting spore suspension was phase bright and >99.9% of vegetative cells removed.

#### Monitoring the initiation of spore germination

The initiation of spore germination was monitored aerobically at 600 nm (the initiation of *C. difficile* spore germination is unaffected by the presence of oxygen). *C. difficile* spore germination was initiated by suspending spores in 50 mM HEPES (pH 7.5), 100 mM NaCl, 100 mM glycine, 10 mM taurocholate and 19% or 38% w / v of sucrose or trehalose or sorbitol. Prior to germination,

spores were heat shocked for 30 min at 65 °C and then placed on ice. Then, 5 µl of spores was diluted into 995 µl of germination buffer (above), mixed, and the change in optical density at 600 nm (OD<sub>600</sub>) was measured over time.

#### Monitoring DPA release

DPA release was monitored in real-time using terbium fluorescence, as described previously [101, 179]. Briefly, an opaque, 96-well plate was prepared with the 125 µl of the germination solutions (see above) supplemented with 800 µM TbCl<sub>3</sub>. A 5-µl sample of a spore suspension (OD<sub>600</sub> of 60) was added to each well, and the DPA release was monitored using a Molecular Devices Spectramax M3 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) [excitation, 270 nm; emission, 545 nm; cutoff, 420 nm (appropriate wavelengths for the DPA-Tb<sup>3+</sup> complex)].

#### Protein extraction and Western blotting

Spores were allowed to germinate in germination buffer (above). NuPAGE soluble proteins (e.g. SleC) were extracted from 2 × 10<sup>9</sup> / ml purified spores, as described previously [101]. Proteins were separated by SDS-PAGE and then transferred for 1.5 hours at 0.75 Amp to an Immobilon-P PVDF 0.45 µm membrane (Millipore). Subsequently, the membrane was blocked for 1 hour at room temperature in Tris-buffered saline (TBS) supplemented with 1% (vol / vol) Tween\*20 (TBST) and 5% dried, skimmed milk. The membrane was then



incubated at room temperature for 1 hour with rabbit anti-SleC antisera. After incubation with the primary antibody, membranes were washed thrice in TBST for 20 minutes each. The membranes were then labeled with goat anti-rabbit IgG (Life Technologies) for 1 hour at room temperature. The membranes were again washed as described above and then exposed to CN/DAB in peroxide substrate buffer. To stop reaction, membranes were rinsed with water and photographed under white light.

#### Assaying cortex fragment release by germinating spores

Cortex fragments were detected according to the method previously reported [179, 182]. Briefly, *C. difficile* spores were heat activated, as described above, and stored on ice until use. A pre-inoculation 1.0-ml sample was drawn to serve as a blank for cortex fragment detection and a separate 100- $\mu$ l sample was taken as a blank for measuring DPA release. A target spore density ( $OD_{600}$ ) of  $\sim 3.0$  yielded the best results for detecting cortex fragments except for MBF02 spores which required a spore density of  $\sim 3.5$ . A 1.1 mL zero time point sample was taken immediately after the addition of spores to the germination buffer and centrifuged for 1 min at  $14,000 \times g$ . Then, 1.0 ml of this sample was transferred to a fresh tube for cortex fragment analysis, and 100  $\mu$ l was taken to monitor the amount of DPA released. This procedure was repeated at selected time points until the experiment was completed. After all time point samples were collected, the samples were frozen at  $-80^{\circ}\text{C}$  and lyophilized.

Lyophilized samples were then analyzed for cortex fragments as previously described [182].

### Statistical analysis

Data points represent the mean from three independent experiments and error bars represent the standard error from the mean. Statistical analysis between time points, where indicated, was performed using a two-tailed Student's *t* test.

## **Results**

### Measuring DPA content of SpoVAC deficient spores

In a previous study, we determined that cortex degradation precedes DPA release and hypothesized that SpoVAC may trigger the SpoVA channel to release DPA in a mechanosensing fashion [179]. Recently, a mutation in *spoVAC* was shown to affect packaging and release of DPA from the *C. difficile* spore [180]. Using the CodA-based allelic exchange system [181], we created a mutant strain in which the *spoVAC* sequence was truncated where only the first 30 base pairs and last 30 base pairs of *spoVAC* were present. Spores were prepared from wild-type *C. difficile* R20291, *C. difficile* MBF02 ( $\Delta$ *spoVAC*) and *C. difficile* MBF02 pMB15 (*pspoVAC*). The DPA content of these spores was measured by boiling the spores, a condition that artificially releases the stored DPA [98]. Similar to the results of a recent study [180], spores derived from the *spoVAC* mutant strain contained approximately 1% of the DPA content that is

found in the wild-type strain (Figure 14) and this could be complemented by expressing *spoVAC* *in trans*. These results confirm the prior observations that *spoVAC* is important for packaging DPA during spore formation [180].

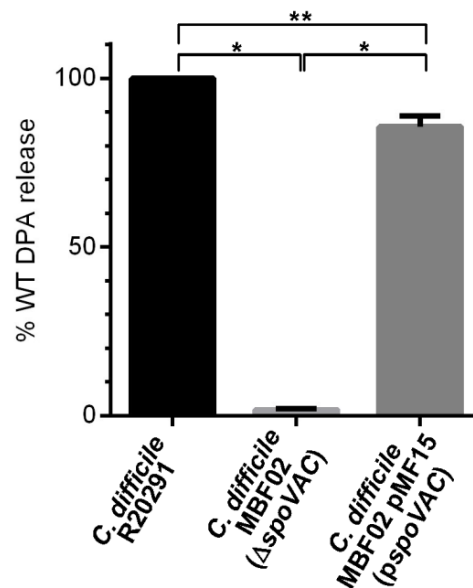


Figure 14. SpoVAC is important for DPA packaging. Equal amounts of spores derived from *C. difficile* strains R20291 (WT), MBF02 ( $\Delta$ *spoVAC*) and MBF02 pMF15 (*pspoVAC*) were boiled for 15 minutes and the amount of DPA was quantified using Tb<sup>3+</sup> fluorescence. The data represent the average from three independent experiments and the error bars represent the standard deviation from the mean. All values are reported as a percent of *C. difficile* R20291 release. \* =  $P < 0.001$ ; \*\* =  $P < 0.05$

### High sorbitol concentrations delay the onset of *C. difficile* spore germination

To test the effects of osmolytes on *C. difficile* spore germination, we measured the change in OD<sub>600</sub> nm of spores suspended in either buffer supplemented with TA and glycine or buffer supplemented with TA, glycine and osmolyte (either sorbitol, trehalose or sucrose). The OD germination assay is a simple method for observing how germination proceeds on the whole (the change from a phase bright spore to a phase dark spore) but does not give detailed information about individual steps (e.g., DPA release or cortex degradation). When *C. difficile* R20291 spores were suspended in buffer supplemented with both TA and glycine, a rapid decrease in the OD<sub>600</sub> of the suspension was observed. However, when *C. difficile* R20291 spores were suspended in buffer supplemented with TA, glycine and increasing amounts of sorbitol (0%, 19% and 38%), the wild-type spores demonstrated a significant delay in the drop in OD<sub>600</sub> (Figure 15A), suggesting that the increasing osmolyte concentration blocked or slowed *C. difficile* spore germination.

To understand if the above observations were due to sorbitol alone or if other osmolytes could substitute for sorbitol, we measure germination in either trehalose (Figure 15B) or sucrose (Figure 15C). As observed for germination in the presence of sorbitol, *C. difficile* spores germinating in the presence of 19% trehalose or sucrose lead to a small delay in germination. However, *C. difficile* spore germination was significantly delayed in the presence of 38% trehalose or sucrose (Figure 15B and Figure 15C, respectively). The results suggest that *C.*

*difficile* spore germination can be delayed under increased osmolyte concentrations.

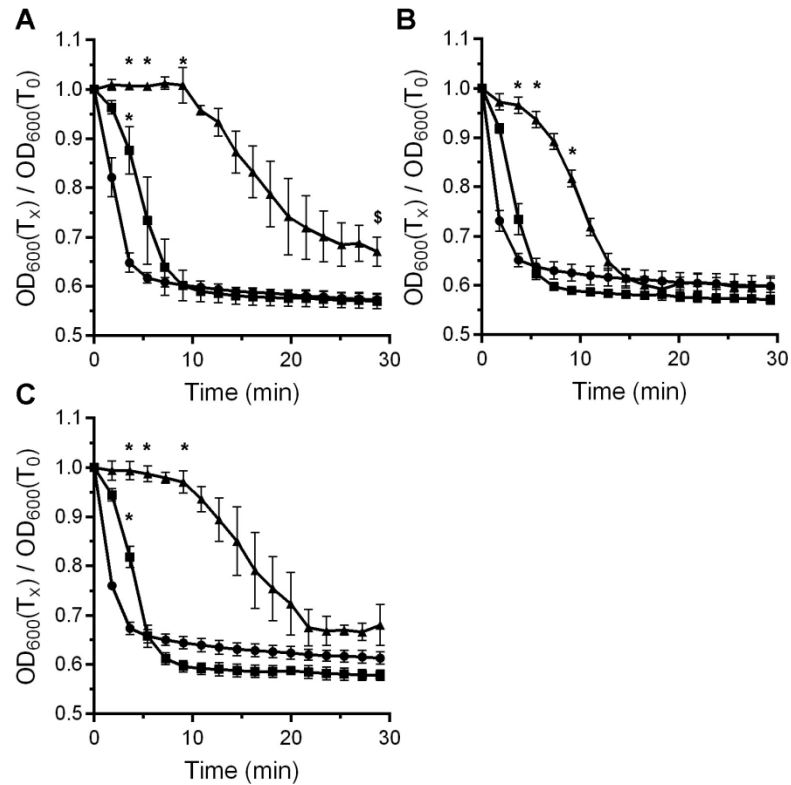


Figure 15. Osmolytes delay germination by *C. difficile* spores. *C. difficile* R20291 spores were germinated in buffer supplemented with taurocholate and glycine in the absence of sorbitol (●) or in buffer supplemented with 19% (■) or 38% (▲) osmolyte. (A) sorbitol; (B) trehalose; (C) sucrose. For clarity, every 5<sup>th</sup> data point is plotted and the data represent the averages from three independent experiments. Error bars represent the standard deviations from the mean. \* =  $P < 0.01$ ; \$ =  $P < 0.05$

### Osmolytes block DPA release during *C. difficile* spore germination

To understand if the osmolyte-mediated delay in germination by *C. difficile* spores was specific to the OD assay or if we would observe similar observations for DPA release, we monitored DPA release by germinating spores in the presence of  $\text{TbCl}_3$  [159, 183]. During germination, the released DPA complexes with  $\text{Tb}^{3+}$  resulting in fluorescence of the lanthanide metal. Thus, DPA release by germinating spores is measured in real time by monitoring DPA-dependent  $\text{Tb}^{3+}$  fluorescence. *C. difficile* R20291 spores were added to buffer supplemented with TA and glycine alone or TA, glycine and sorbitol and DPA release was monitored as a readout for germination (Figure 16A). In the absence of sorbitol, spores rapidly release their stored DPA and an increase in  $\text{Tb}^{3+}$  fluorescence is observed (Figure 16A). Interestingly, spores delayed the release of DPA in the presence of sorbitol. We found that 38% sorbitol lead to a delay of ~8 minutes over the course of the experiment (Figure 16A). To understand if DPA release could be blocked / delayed by other osmolytes, spores were germinated as above but sorbitol was substituted with trehalose (Figure 16B) or sucrose (Figure 16C). Both trehalose and sucrose delayed DPA release by germinating *C. difficile* spores.

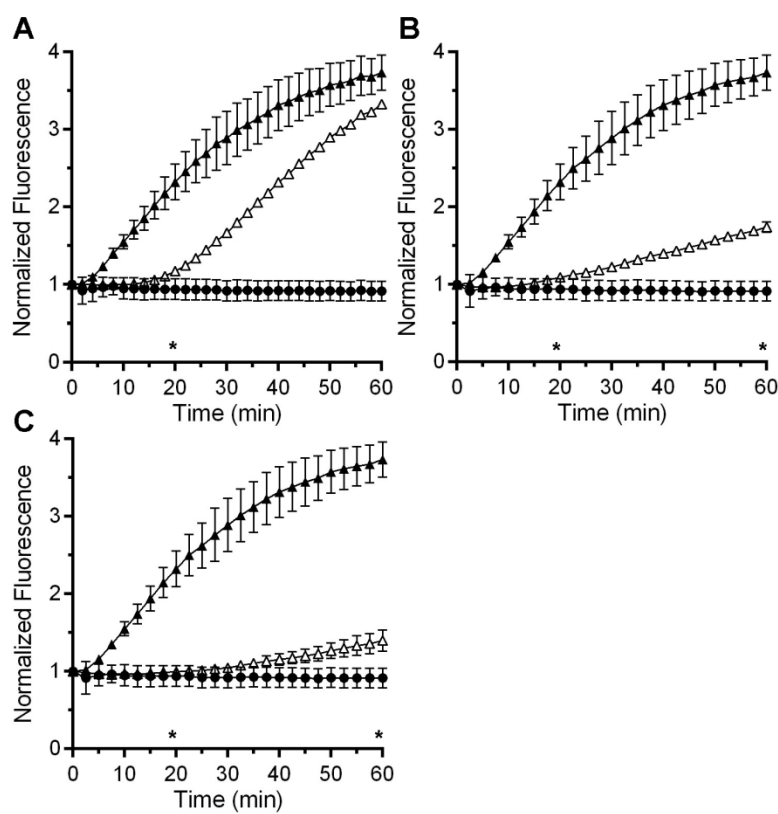


Figure 16. Osmolytes delay DPA release by *C. difficile* spores.

*C. difficile* R20291 spores were suspended in buffer containing terbium chloride and glycine alone (●), supplemented with taurocholate (▲) or supplemented with taurocholate and 38% (Δ) osmolyte. (A) sorbitol; (B) trehalose; (C) sucrose. For clarity, every 5<sup>th</sup> data point is plotted and the data represent the averages from three independent experiments. Error bars represent the standard deviations from the mean. \* =  $P \leq 0.01$ . Statistical significance between TA + glycine conditions and 38% sorbitol conditions was only tested at the 20 and 60 minutes time points.

Prior reports have suggested that there is heterogeneity between different *C. difficile* strains in terms of their response to germinants [101, 164, 165].

Though the rates and efficiencies with which *C. difficile* spores germinate in

response to bile acids are reported, the mechanisms underlying the response to germinants are likely conserved among strains [101, 179, 184]. To ensure that our observations that high osmolyte concentrations delay the release of DPA from germinating *C. difficile* spores are not due to a strain-specific phenotype, we determined if the *C. difficile* M68 strain would yield similar results. DPA release by *C. difficile* M68, ribotype 017, spores was delayed by increased sorbitol or trehalose or sucrose concentrations (Figure 17). These results suggest that the effects of high osmolyte concentrations on *C. difficile* spore germination are not strain specific and that high osmolyte concentrations delay the release of DPA from the core of germinating *C. difficile* spores.



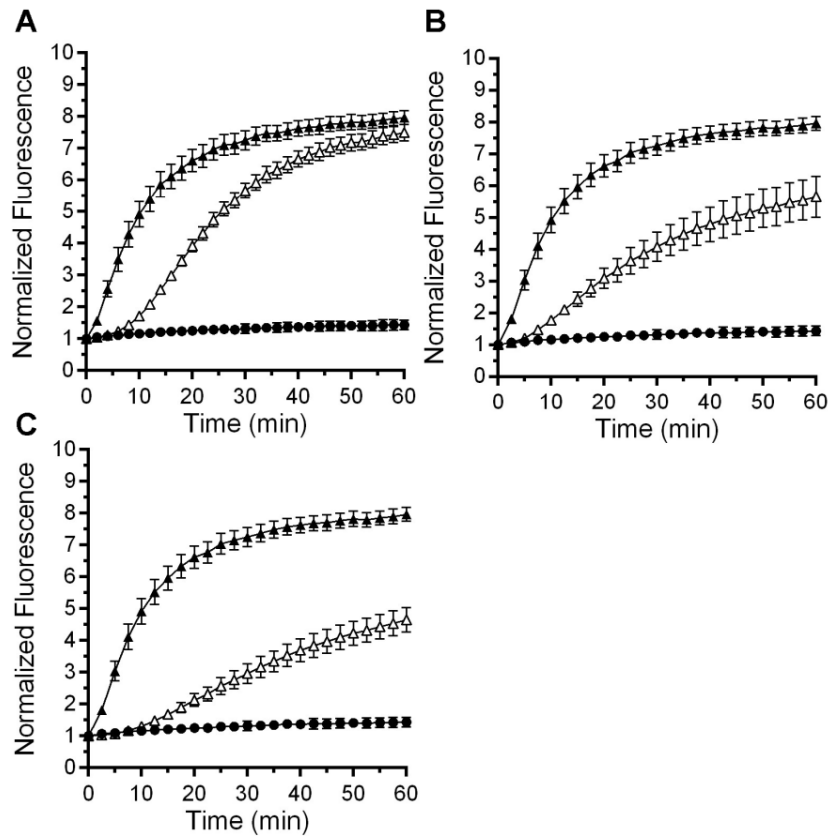


Figure 17. Osmolytes delay DPA release by *C. difficile* M68 spores. *C. difficile* M68 spores were suspended in buffer containing terbium chloride and glycine alone (●), supplemented with taurocholate (▲) or supplemented with taurocholate and 38% (Δ) osmolyte. (A) sorbitol; (B) trehalose; (C) sucrose. For clarity, every 5th data point is plotted and the data represent the averages from three independent experiments. Error bars represent the standard deviations from the mean.

#### Pro-SleC is cleaved to an active form in high osmolyte concentrations

The delay in OD change and in DPA release could be the result of the high osmolyte concentration retarding the rate at which germinants (TA and glycine) interacted with the germinant receptors. To test if the germinants were

still activating germination in the presence of osmolyte, we tested if the SCLE, pro-SleC, was cleaved to its active form (SleC activation is necessary for cortex degradation). *C. difficile* spores were suspended in buffer with glycine only, as a negative control, or in buffer supplemented with TA and glycine with or without 38% osmolyte. Samples were taken at the indicated times and processed for immunoblotting. In the absence of TA, spores do not activate pro-SleC (Figure 18). However, spores rapidly cleave pro-SleC to its active form in response to TA and glycine. Significantly, the presence of osmolyte had no effect on the timing of SleC activation (Figure 18).

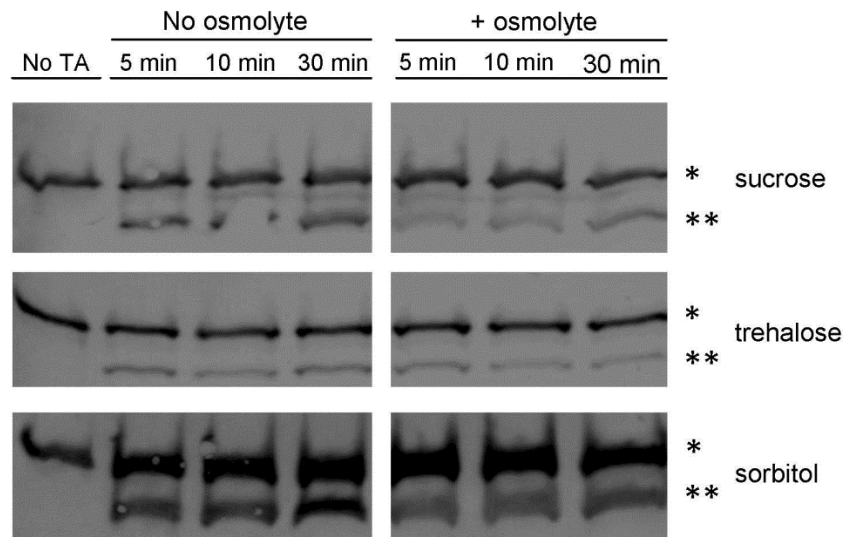


Figure 18. High osmolyte concentrations do not affect SleC activation. Cleavage of pro-SleC (\*) to SleC (\*\*) was assayed in buffer supplemented with glycine or buffer glycine and taurocholate with or without 38% osmolyte at 5, 10 and 30 minutes.

### Detecting cortex degradation in the presence of osmolytes

If SleC is activated in the presence of osmolyte, the appearance of cortex fragments in the germinant solution should be readily detected. To detect these fragments, we made use of a colorimetric assay that we, and others, have used to quantify the presence of reducing sugars [81, 178, 179, 182]. This assay detects the presence N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues (reducing sugars) released during the degradation of cortex peptidoglycan [182]. Purified spores were germinated in the presence of TA and glycine and were sampled every 2 minutes for the presence of both cortex fragments and DPA (Figure 19). The results for *C. difficile* R20291 spores were similar to those previously reported for *C. difficile* strain UK1, where cortex degradation and DPA release seemingly occur simultaneously (Figure 19A) for a wild-type strain [using mutant strains, we previously observed that cortex degradation precedes DPA release [179]]. When we tested cortex degradation and DPA release in buffer supplemented with 38% sorbitol with TA and glycine (sorbitol was the only osmolyte tested in this assay because it is the only compound tested that does not generate a reducing end), the release of cortex fragments occurs in a similar manner as in the absence of sorbitol while DPA release is delayed and only begins to be observed after 8 minutes (Figure 19B).

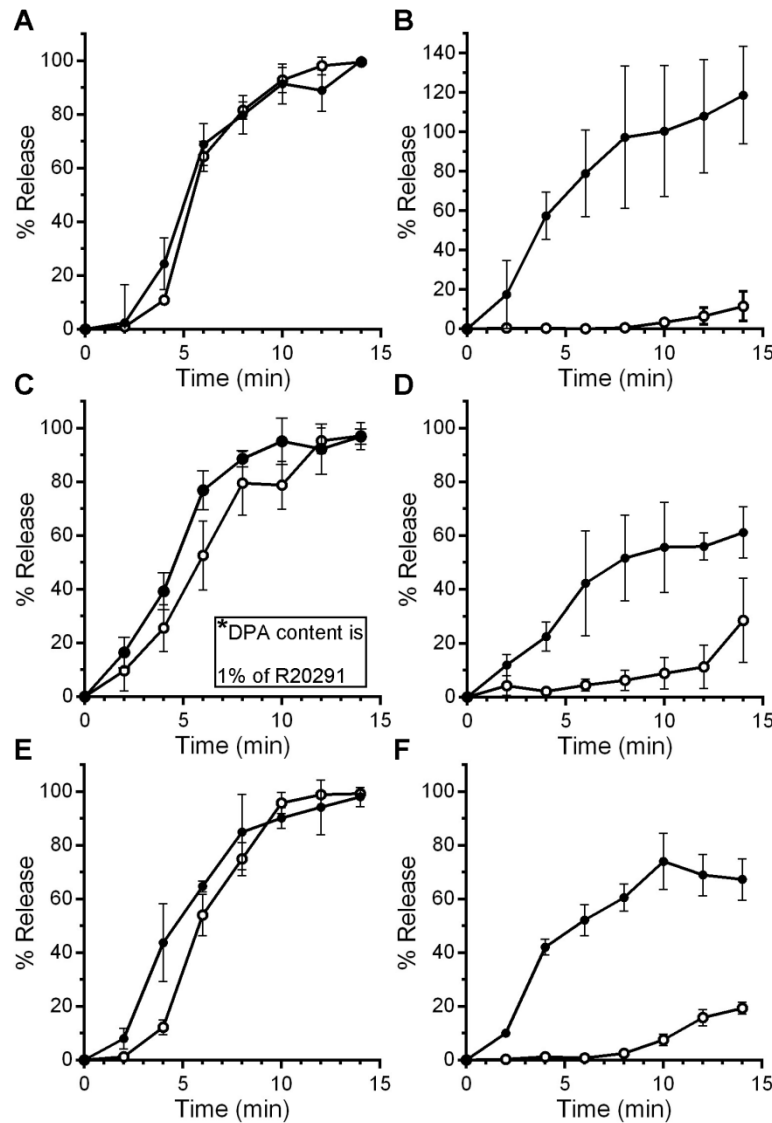


Figure 19. High osmolyte concentrations delay DPA release but not cortex degradation.

Purified *C. difficile* R20291 spores (A and B), *C. difficile* MBF02 ( $\Delta spoVAC$ ) spores (C and D) or *C. difficile* MBF02 pMF15 ( $pspoVAC$ ) spores (E and F) suspended in buffer supplemented with taurocholate and glycine and no sorbitol (A, C and E) or the same buffer supplemented with 38% sorbitol (B, D and F). At the indicated time points, samples were taken for the amount of (●) cortex fragments and (○) DPA. Values for all graphs are normalized to average, maximum, amount of cortex or DPA released by each strain in the absence of sorbitol. The data represent the averages from three independent experiments and error bars represent the standard deviation from the mean.

When germinated in TA with glycine, *C. difficile* MBF02 ( $\Delta spoVAC$ ) spores released cortex fragments similarly to the wild-type strain (Figure 19C). DPA release by this strain also occurs, however the total amount of DPA in the MBF02 strain is only 1% of that found in the R20291 strain (Figure 14). When the *spoVAC*-mutant spores were germinated in the presence of 38% sorbitol, the release of what little amount of DPA is present is delayed to the very end of the time period, while cortex degradation remains largely unaffected (Figure 19D). These observations could be complemented by expressing *spoVAC* *in trans* from a plasmid (Figure 19E and Figure 19F).

#### Altering osmolyte concentration affects DPA release during germination

If DPA release is dependent on both cortex degradation and on a mechanosensing mechanism, changing the osmolyte concentration during germination should result in a marked change in the rate of DPA release. To test the effects of an osmotic downshift on DPA release by germinating spores, spores were allowed to germinate for 5 minutes in one volume of 38% sorbitol-containing germination buffer (containing TA and glycine). Then, either two volumes of germination buffer with sorbitol (leading to no change in osmotic conditions) or two volumes of the germination buffer without sorbitol (leading to an osmotic downshift) were then added to the well. Germination was then monitored under these new conditions. In samples that had sorbitol-containing germination buffer added, DPA release was delayed, as expected – the osmotic

strength of the solution did not change (Figure 20). However, diluting the osmolyte to 12.7% (a three-fold dilution) resulted in a rapid release of DPA from the core (Figure 20). As controls, samples where the spores were germinated in germination buffer alone or germination buffer supplemented with 38% sorbitol for the duration of the experiment were included. As expected, in the control samples, DPA was released in the no-sorbitol-containing germination buffer while the sorbitol-containing germination buffer blocked DPA release (Figure 20). These results suggest that DPA release during germination by *C. difficile* spores occurs through a mechanosensing mechanism that is dependent on degradation of the spore cortex layer.

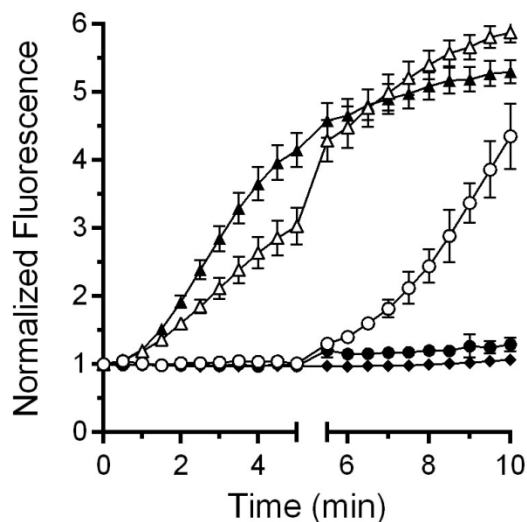


Figure 20. Altering osmotic conditions during germination influences DPA release.

Purified *C. difficile* R20291 spores were suspended in buffer supplemented with taurocholate and glycine alone (▲) or the presence of with 38% sorbitol (◊) and allowed to germinate for 10 minutes. In a separate experiment, spores were germinated for 5 minutes in buffer supplemented with taurocholate and glycine alone (Δ) or with 38% sorbitol (○). These samples then were diluted with 2 volumes of buffer supplemented with taurocholate and glycine alone. Finally, a separate set of spores were germinated for 5 minutes in buffer supplemented with taurocholate, glycine and 38% sorbitol (●) and then 2 volumes of 38% sorbitol-containing germination buffer were added and germination continued for another 5 minutes.

## Discussion

The release of DPA from the core of a spore is a crucial step during spore germination. However, the mechanisms of DPA release by germinating spores are poorly understood. Here, we have reaffirmed that SpoVAC plays a critical role in DPA import into the core during spore formation (Figure 14) [180].

Unfortunately, our current understanding of the mechanism of DPA release and

SpoVAC activity prevents the separation of DPA packaging during *C. difficile* spore formation and DPA release during germination. Thus, to begin to understand the mechanisms of DPA release by germinating *C. difficile* spores, we raised the concentration of osmolytes in the germination solution. The increase in osmolyte concentration led to a marked delay in DPA release but not a delay in the activation of SleC, a key step in germination (Figure 16 and Figure 18). By using an assay that detects the presence of reducing sugars (*i.e.*, cortex fragments) [182], we were able to compare when the cortex was degraded relative to DPA release in conditions where the osmotic strength of the medium was increased. Under these conditions, DPA release is delayed, though cortex degradation is unaffected (Figure 19). Because cortex degradation precedes DPA release during *C. difficile* spore germination, these results suggest that cortex degradation relieves the constraints put in place by the cortex on the core leading to the mechanosensing protein, SpoVAC, permitting DPA release.

*spoVAC* is conserved across most endospore-forming organisms and the proteins encoded by the *spoVA* operon play important roles during spore-formation and germination [52, 173, 180, 185]. In *B. subtilis*, formation of a dormant, heat-resistant spore is dependent on 5 of the proteins encoded in the *spoVA* operon: *spoVAA*, *-AB*, *-AC*, *-AD*, and *-AEb*. SpoVAF and SpoVAEa localize to the inner membrane of the spore and have minor roles during spore germination [55]. Defects in these proteins reduce germinant-dependent germination but do not affect non-nutrient-mediated spore germination (*e.g.*,



exogenous DPA) [55]. In *B. subtilis*, the release of DPA from the core triggers degradation of the spore cortex [66]. For DPA to be released from the spore core, a signal first must be transmitted from the germinant receptors to the SpoVA protein. SpoVAF and SpoVAEa may play accessory roles during germination to efficiently receive and transmit the germination signal from the GerA or GerB/GerK to the SpoVA complex and permit DPA release. Importantly, *C. difficile* does not encode *ger*-type germinant receptors nor does *C. difficile* encode *spoVAF* but it does encode *spoVAE* and *spoVAD* homologs [150]. The roles of SpoVAE and SpoVAD during *C. difficile* spore germination are unknown. However, based on work in *B. subtilis*, it is likely that SpoVAD is essential for DPA packaging during sporulation [54].

Of the *spoVA* encoded proteins, SpoVAC is nearly universally conserved among spore formers [185]. Though the number of proteins encoded by the *spoVA* operon varies between organisms, nearly all encode *spoVAC*. However, there are a few exceptions. In the Clostridiales, *Bryantella formatexigens* only encodes *spoVAA* and *spoVAB* and *Carboxydibrachium pacificum* does not encode any apparent *spoVA* homologs [185]. Because of the nearly-universal conservation of *spoVAC* among spore-formers, this suggests that mechanosensing plays an important role during spore germination.

Mechanosensing during spore germination was previously studied during germination by *B. subtilis* spores [168-170]. The authors found that *B. subtilis*  $\Delta mscL \Delta mscS$  germinated similarly to a wild type strain in response to L-alanine

and dodecylamine [170]. Thus, the proteins required for osmotic stability during growth play little to no role during germination by *B. subtilis* spores. However, the mechanosensing SpoVAC protein plays an important role during spore formation and germination [52, 180].

The data supporting SpoVAC as a mechanosensing protein is based, partly, on the ability of recombinantly expressed SpoVAC-myc-6His to protect *E. coli* from an osmotic downshift [53]. The authors found that SpoVAC-myc-6His protected *E. coli* to a similar extent as MscL, a well-studied mechanosensing membrane protein [186, 187]. The authors further characterized the protein using conductance studies and found that, when recombinantly expressed in *E. coli* and embedded in lipid vesicles, SpoVAC has a pore size of 4.6 Å [53]. This pore size should be large enough to accommodate DPA (a planar molecule with dimensions of 5.2 Å in length x 3.5 Å in width) but the authors mention that the vesicles may not recapitulate the lipid content or the hydration state of the inner spore membrane and this could affect gating of the protein [53]. Prior work has suggested that inner spore membrane to be in a gel-like state (or in a state that prevents mobility of protein within the membrane) [188]. An interesting hypothesis is that upon the initiation of cortex degradation, local changes at the inner membrane are observed which triggers SpoVAC-mediated DPA release from the core. Subsequently, the change in hydration state of the inner spore membrane at the site of DPA release results in the signal propagating to the surrounding SpoVAC proteins. The prediction of such a system would be that

high osmolyte concentrations could only block DPA release for so long before a few SpoVAC proteins become activated, randomly. This then would trigger the rest of the SpoVAC proteins to open and allow DPA to escape the core. In support of this hypothesis, we find that sorbitol or trehalose or sucrose do not permanently block the release of DPA from germinating spores (Figure 16 and Figure 17). These osmolytes only delay the release of DPA from the germinating spore. For sorbitol-containing germination buffer, the rate with which DPA is released after the initial delay is similar to the rate in the absence of sorbitol (Figure 16A). This would suggest that the SpoVAC channel activity is not affected by sorbitol, or water activity, in our assays. In this experiment, sucrose functioned better than trehalose and trehalose functioned better than sorbitol but DPA release still occurs and the rate of DPA release begins to increase at later time points. Importantly, though, what little DPA is present in the *C. difficile*  $\Delta spoVAC$  strain is still released during spore germination [(Figure 19C), though the value is presented as 100% release, the data are normalized between +/- sorbitol conditions and not between strains. The  $\Delta spoVAC$  mutant has a low amount of DPA (Figure 14) [180]]. This might suggest that another protein could function in an accessory role to permit DPA release in response to the change in osmolarity at the inner spore membrane upon cortex degradation or that the membranes leak DPA in the absence of SpoVAC.

Understanding the process of germination and how DPA release is triggered is likely more straightforward in *C. difficile* than in other spore-forming

organisms. Because only *spoVAC*, *spoVAD* and *spoVAE* are encoded in *C. difficile*, the mechanism of DPA release likely does not involve the interaction with other factors (e.g., germinant receptors). In *C. difficile*, the bile acid germinant receptor, CspC, likely transmits the bile acid signal to CspB which then cleaves pro-SleC to an active, cortex-degrading, form [98, 99, 189]. Activated SleC then begins degrading cortex allowing expansion and SpoVAC-mediated DPA release, core hydration and subsequent outgrowth of a vegetative cell. The absence of germinant receptor in the inner spore membrane simplifies the mechanism of DPA release but may result in less specificity and a tendency for increased amount of spontaneous DPA release. Interestingly, *Clostridium perfringens* encodes a *spoVA* system similar to that of *C. difficile* and also encodes functional germinant receptors [79, 101, 167, 185]. How DPA release is signaled in this organism is unknown but its understanding would be of important value for understanding how the SpoVAD, SpoVAC and SpoVAE interact in such a system.

## CHAPTER V

### SUMMARY OF FUTURE DIRECTIONS OF *C. DIFFICILE* GERMINATION RESEARCH

#### **Introduction**

With the advent of improved genetic tools, *C. difficile* research has begun to target specific genes for clean deletions. Previously, the limited genetic toolbox only allowed for the disruption of individual genes based on the ability to insert antibiotic resistance genes, e.g. the TargeTron system. This system has many limitations, including potential polar effects and off-target insertion events. Recently, allelic exchange techniques have been developed, allowing for the clean deletion of target genes, as with *spoVAC* in Chapter IV. Currently, CRISPR techniques are being developed in our lab that will allow improved gene deletions.

With these improved tools, the future direction of this research is to first determine the role that *spoVAD* and *spoVAE* play in spore formation, DPA packaging, germination and DPA release. *SpoVAD*, based upon research in *B. subtilis*, is expected to play a role in packaging of the DPA during spore formation. Removal of *spoVAD* is expected to result in spores with less DPA. The role *SpoVAE* plays is less clear. It could be associated with pore opening, i.e. the observed result (based on DPA release) of all *SpoVAC* pores appearing to open collectively. Alternatively, *SpoVAE* could be associated with allowing of

SpoVAC, functioning in a manner similar to the hypothesized role SpoVAEa and SpoVAF that affects the rate of DPA release in *B. subtilis*.

When compared with the functions of the *B. subtilis* *spoVA* operon, the role of the smaller *spoVACDE* of *C. difficile* may not be the same. An interesting experiment will be replacing the entire *spoVA* operon in *B. subtilis* with the *C. difficile* *spoVACDE*. This could result in a *B. subtilis* spore that would still package DPA but release of DPA would be affected. Presumably, the *C. difficile* SpoVAC would be incapable of interacting with the Ger proteins. Germination of these spores would then only occur when CwlJ is activated by exogenous DPA. In this pathway, CwlJ would degrade the cortex, allowing the core of the *B. subtilis* spore to swell and then the *C. difficile* SpoVAC-related pore would open, allowing DPA release. The purpose of these experiments would be to see if *C. difficile*-like germination could be reconstituted in *B. subtilis*.

In tandem with understanding the process of DPA packaging and release, research will also focus on the transition in the spore occurring as the core rehydrates and the spore becomes a vegetative cell. During this stage, termed ripening, metabolic functions begin to resume within the spore. This is an important stage, as the spore has lost its resistance properties but has not fully resumed the ability to mount a proper stress response. It is at this point, when the DPA is released and the core rehydrates, translation of stored RNA is believed to initiate. Understanding these initial products could be important for targeting infections at this vulnerable stage.

Another area of on-going research related to this dissertation is the role of amino acids in *C. difficile* germination. How the amino acids interact with the germination complex is currently unknown. It is unclear if the amino acid co-germinant interacts directly at CspC with TA or if it is interacting at another location within the germination complex. It may act to assist in the release of members of the germination complex (CspC, CspB, SleC) from GerS or from each other, *i.e.* releasing active SleC from CspBC. Earlier attempts using the similar mutagenesis method that isolated CspC as the bile acid germinant receptor failed to successfully isolate the amino acid germinant receptor.

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